Immunomodulatory and Anti-tumor Activities of *Flammulina velutipes*

by

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ABBREVIATIONS

ADCC	Antibody-dependent Cellular Cytotoxicity
APCs	Antigen-presenting Cells
B-cell	Bursa-derived Cell
BSA	Bovine Serum Albumin
C'	Complement
ConA	Concanavalin A
CTL	Cytolytic T-lymphocyte
ddH_2O	Double distilled water
dH_2O	Distilled water
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
F.V.	*Flammulina velutipes*
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GVB	Gelatin Veronal Buffer
HBSS	Hank's Balanced Salt Solution
HIFCS	Heat Inactivated Fetal Calf Serum
^{3}H-TdR	Tritiated Thymidine
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin

K	Killer
LAL	Limulus Amebocyte Lysate
LC_{50}	Lethal Concentration 50 %
LD_{50}	Lethal Dosage 50 %
mCi	Millicurie
MHC	Major Histocompatability Complex Molecule
MWCO	Molecular Weight Cut Off
MTT	(3-[4,5-Dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide)
NK	Natural Killer
PBS	Phosphate Buffer Saline
PEC	Peritoneal Exudate Cell
PMB	Polymyxin B Sulphate
PPO	2,5-diphenyloxazole
POPOP	1,4-bis[2-(5-Phenyloxazole)] benzene
PSF	Penicillin-Stretomycin-Fungizone solution
rIL-3	Recombinant Interleukin-3
SDS	Sodium Dodecyl Sulphate
S.I.	Stimulation Index
SRBC	Sheep Red Blood Cell
T-cell	Thymus-derived Cell
TIL	Tumor-infiltrating Lymphocyte
TLC	Thin Layer Chromatography
TNS	Tumor Necrosis Factor-containing Serum

TNF	Tumor Necrosis Factor
TSAs	Tumor-specific Antigens
TSTA	Tumor-specific Transplantation Antigen
VDH	Vascular Dilation and Haemorrhage
YW	Yeast Cell Wall

Aim and Scope of This Dissertation

The guiding principle of practitioners of Traditional Chinese Medicine is the maintenance of the natural balance of the human body and its restoration to balance during periods of pathogenic invasions and poor health. Substances, for such curative purpose, are derived from nature. The substances can be animal bodies, organs or tissues, higher plant structures, algae, fungi etc.. Fungi such as *Ganoderma applanatum* and *Ganoderma lucidum* have long been valuable substances in Traditional Chinese Medicine. It has been found that polysaccharides extracted from these fungi were active biological response modifier (BRM) and antitumor agent. Other fungi, including many edible mushrooms such as *Lentinus edodes* were also found to have polysaccharides which possesses BRM and antitumor properties.

In this project, three edible mushrooms which are popular foodstuffs in oriental countries will be screened as a source for the extraction of potential antitumor polysaccharide(s). *Flammulina velutipes*, one of the mushrooms chosen for screening, was selected for the extraction. The bioactive components will be isolated and partially purified from the cell wall of *Flammulina velutipes*. Furthermore, the chemical, structural, immunological and antitumor properties of the extracted components will be elucidated.

Abstract

Many anti-tumor polysaccharides, isolated from fungi, were identified as β-(1→3)-D-glucans. In this project, three types of edible fungus ; *Volvariella volvacea*, *Lyophyllum aggregatum* (*shimeji*) and *Flammulina velutipes*, were screened for the presence of β-(1→3)-D-glucan by aniline blue staining. By the staining method, *Flammulina velutipes* was found to be the richest source of β-(1→3)-D-glucan among the three fungi and was chosen as the target fungus in the project. Furthermore, the β-(1→3)-D-glucan was mainly located at the cell wall rather than in the cytoplasm of the fungus.

After screening , the isolation of β-(1→3)-D-glucan from the cell wall of the mushroom was tried. Cell wall materials of the mushroom was obtained after extensive washing of the lyophylised and powdered fungus with several types of solvent (ddH_2O, chloroform-methanol mixture, acetone and ddH_2O). β-(1→3)-D-glucan was first extracted from the cell wall by hot water extraction (121°C, 30 min.). The hot water extracted residues was then treated with alkaline-urea mixture (65 °C, 30 min.) to release the remaining β-(1→3)-D-glucan in the hot water extracted cell wall. The extracted β-(1→3)-D-glucans were neutralised first with acetic acid and then dialysed with dH_2O to retain macromolecules of molecular weight > 3.5 kD. The hot water extracted β-(1→3)-D-glucan was named FH and the alkaline extracted β-(1→3)-D-glucan was named FAI. The FAI fraction was further separated into the soluble fraction (SFAI) and the insoluble fraction (IFAI). The isolated FH, FAI and SFAI fractions were found to be able to trigger a unique type of inflammation, VDH (Vascular Dilation and Haemorraghe) response. The response was reported to have a high correlation with the antitumor activity of the polysaccharides. On this basis, FH and SFAI were selected for further studies. Both FH and SFAI fractions were found to be similar in their physical properties. For example, they are both highly viscous and have molecular weight estimated ≥ 300 kD by gel

permeation chromatography. Chemical analysis showed that both FH and SFAl contain mainly glucose. The identity of both FH and SFAl was confirmed to be β-(1→3)-D-glucan by enzyme (a β-(1→3)-D-glucanase isolated from a brown algae; *Laminaria* sp.) digestion analysis. However, FH and SFAl were found to possess different secondary structure. By the Congo Red dye method, FH was found to possess a triple helical structure and SFAl possess a single helical structure.

Both FH and SFAl were found to have no significant cytotoxic effect when using Brine Shrimp as indicator. FH and SFAl also have no direct cytotoxic effect on murine tumor cell lines such as Sc-180 and PU5-1.8. When the mediation of the regression of transplantable tumors (Sc-180 and PU5-1.8) on mice was tested both FH and SFAl caused more than 90 % of tumor regression. The regression was suspected to be caused by the augmentation of murine immune system by FH and SFAl. In the *in vitro* system, the activation of B-lymphocyte, T-lymphocyte and macrophage were tested. It was found that only the proliferation of B-lymphocyte was slightly enhanced by FH . In the *in vivo* system, both FH and SFAl were shown to activate both T-cell and macrophage, and an increase in serum interferon-γ level. Other than these activities, FH and SFAl when administered into mice can mediate an increase in serum complement level and FH can mediate the secretion of tumor necrosis factor (TNF). From the results, it was concluded that FH and SFAl can enhance the regression of some transplantable tumors in mice. The enhancement of the regression was not due to a direct cytotoxic effect. Instead, the killing was mediated through the augmentation of the murine immune system.

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INTRODUCTION

1.1 INTRODUCTION

Neoplastic growth is a result of the transformation of normal cell into a state of uncontrolled cell division. The transformation may be induced by various chemicals, physical and viral agents. Benign neoplasm when left unnoticed may convert into fatal malignant tumor. The prevention of neoplastic growth in our body is achieved by our immune defence mechanisms. It was proposed that tumor do possess particular antigens , such as tumor-specific transplantation antigens (TSTAs) and tumor-specific antigens (TSAs) (Schreiber *et al*, 1988), which can be recognized and destroyed by our immune system.

The treatment of cancer has long been relied on surgery, radiotherapy and drug therapy. Although these therapeutic strategies are common in clinical practices, they suffer from a lot of limitations and disadvantages. Recently, two new branches of cancer therapy evolved—gene therapy and immunotherapy. These therapies seem to be the most attractive cancer therapy. It is because these therapies cause less side effects than other types of therapy.

Immunotherapy includes antibody therapy, adoptive cellular immunotherapy, cytokine therapy and stimulation of immune effectors. The stimulation of immune effectors can be done by a lot of natural substances. In my M. Phil project, I aimed to extract potential anti-tumor polysaccharides, β-(1→3)-D-glucan(s), from the cell wall of *Flammulina velutipes*, which kill tumor via stimulating the host immune effectors.

1.2 TUMOR BIOLOGY

There exist DNA sequences, the oncogenes (Hunter, 1984), in the normal cell nuclei when altered can cause neoplastic change. Oncogenesis may be induced by chemicals such as carcinogenic hydrocarbon methycholanthrene etc., physical agents such as UV, X-, γ-irradiation etc., and viral agents (Bishop, 1985) such as AIDS virus etc.. Oncogenesis may occur as a result of over expression of the unaltered genes (quantitative alternation) or as a result of a mutation which causes the gene to produce a new protein (qualitative alternation). These agents always cause alternation at chromosomal level (Croce and Kleiu, 1985). Sometimes, the alternation is specific in nature, e.g. the 9:22 translocation in chronic granulocytic leukemia (Philadelphia chromosome) and the 8:2, 14 or 22 translocation in Burkitt's lymphoma, a tumor of B-lymphocytes. In the Burkitt's translocation, the *c-myc* oncogene is transferred to a chromosomal region which normally codes for immunoglobulins. Some of the proteins produced by oncogenes in the initiation or maintenance of malignancy have been discovered to have biological effects which may, at least partly, explain the neoplastic process. Some are extremely similar to normal growth factors (e.g. platelet-derived growth factor, epidermal growth factor), or their cell surface receptors activates enzymes, such as kinases, and can thereby induce cell division.

Malignant tumors usually show an increased mitotic activity. However, malignant cells do not always multiply faster than their normal counterparts and a tumor results because of an imbalance between cellular proliferation and cell loss. In a tumor, there is a variable proportion of malignant cells and a substantial proportion of non-malignant stromal or reactive cells. Only a proportion of the malignant cells component within cancer have the capacity for indefinite self-replication. They are called the stem cells and are the targets for treatment with radiotherapy or drug therapy. The rate of tumor growth depends on the size of the growth fraction, the proportion of malignant cells

proceeding through the cell cycle to mitosis at any one time, and also on the cell cycle time.

Due to the occurrence of mutation in active proliferating cells, heterogeneity increases as the tumors grows. The heterogeneity includes variations in cellular appearance, hormone receptor concentrations, and variations in response to treatment in different metastases. This heterogeneity is thus a substantial block to successful treatment. Radiotherapy, hormone therapy, cytotoxic chemotherapy and monoclonal antibody therapy often fail because of the presence of a small number of cells which are resistant to attack.

Other than heterogeneity, there is a property of malignant tumor called metastatic spread (Nicolson, 1979) that makes cancer difficult to be cured. Metastatic spread is a complicated process which occurs more frequently with undifferentiated, rapidly growing tumors. Only a very small proportion of shed cells are capable of eventual development into distant tumors. For successful spread, cells have to breach the vascular basement membrane in a target organ and then proliferate in a foreign micro-environment. The breaching of basement membrane may involve, collagenase and other digestive enzymes.

1.3 THE DEFENCE MECHANISMS

All animals have defence mechanisms that provide protection against pathogens such as chemicals, virus, bacteria, fungi, protozoa, helminthes and foreign tissues. The most complicated and well developed defence mechanisms are the human defence mechanisms. The defence mechanisms of our body can be non-specific or highly specific.

1.3.1 NON-SPECIFIC DEFENCE MECHANISMS

Non-specific defence mechanisms, innate immunity (Roitt, 1988), are directed against a multitude of foreign agents by barriers, preventing their entrance into our body, or by destroying them quickly if they do penetrate the body's barriers. The major barrier is the skin which is impermeable to most infectious agents. Furthermore, the acidic secretions of the skin suppress the growth of bacteria. The inner surface of our body is lined with membranous layer covered with mucus. The mucus together with trapped bacteria is removed by ciliary movement, coughing and sneezing. Secretions from glands or the barriers reinforce the barrier function such as acid in gastric juice, spermine and zinc in semen, lactoperoxidase in milk and lysozyme in tears, nasal secretions and saliva. Gut microbial flora which can suppress the growth of other microorganisms also reinforce the barrier function.

The destruction of invading pathogens is achieved by agents such as phagocytic cells, complement, acute phase proteins, interferons, natural killer cells and eosinophils. Any invaded circulating microorganisms are ingested by phagocytes, polymorphonuclear neutrophils and macrophages, and were killed inside the phagocytes. Alternately, the circulating microorganisms may activate the blood complement proteins, through the alternate pathway, which drill holes on the microorganism and cause lysis. Acute phase proteins, C-reactive protein, fibrinogen, C9, Factor B etc., increase during infection may improve our general defences. The level of the anti-viral agents; interferons (α-interferons, β-interferons and γ-interferons) increases during viral infection. The interferons can suppress viral replication and its spreading. Natural killer cell (NK cell) is a type of large granular lymphocytes which help defence viral infection by killing virally infected cells and other pathogenesis. Eosinophils are leucocytes which play major defence role in helminthe infection. All the agents described kill invaders in a non-specific manner.

1.3.2. SPECIFIC DEFENCE MECHANISMS

The specific defence mechanisms or the immune response, unlike non-specifc defence mechanisms, when activated are tailor-made to particular type of pathogenesis and is highly effective in removing the pathogen. In general, there are two main types of immune responses; the antibody mediated immune response and the cell-mediated immune response.

For the immune responses to be activated, any invaders should be processed before they can be recognized by either the antibody- or cell-mediated immune response called antigen presentation (Janeway, 1993). The antigen presentation requires antigen-presenting cells (APCs) such as macrophages, dendritic cells, B-cells etc.. During the process, invaders or antigens are first required to bind to APCs. The membrane bound antigens are then internalised through phagocytosis, receptor-mediated endocytosis or pinocytosis to form cytoplasmic vesicles called endosomes. In endosomes, the antigens are digested into fragments by enzymes, and digested fragments are made to associate with major histocompatability complex molecule (MHC). This digested antigen fragment and MHC complex is finally migrated to surface membrane of APCs and ready to be presented to other cell types of the immune system such as helper T-cell.

Antibody-mediated immune response is highly effective in removing circulating pathogens. In antibody-mediated immune response, the effector agent is antibody which is produced by B-lymphocytes. Before a resting B-cell can produce antibody effectively, it should be activated. The activation of a resting B-cell and T-cell involve the binding , processing and presenting of corresponding antigen to helper T-cell—the master cell for both antibody- and cell-mediated immune response. After presentation, this helper T-cell is activated to increase its interleukin-2 (IL-2) receptor expression and its IL-2, IL-6 and interferon-γ (IFN-γ) secretion. The secreted IL-2 stimulates the resting B-cell to proliferate and differentiate into a clone of plasma cell. This clone of plasma cell produces

antibody effectively, and the antibody secreted only recognizes antigen that has been presented (the antibody secreted is monoclonal with respect to its antigen binding site). The constant region of the secreted antibody can be defined into five classes namely IgG, IgA, IgM, IgD and IgE. It is the differences among the classes of antibody that allow the antibody call for different killing mechanisms involving complement, NK cells, macrophage, mast cell, neutrophil etc., to destroy the antigen effectively.

Cell-mediated immune response unlike antibody-mediated response is effective in removing intracellular invader (virus), malignant cells and foreign tissues. In the cell-mediated immune response , the effector agent is cytotoxic T-lymphocytes (CTLs). The activation of pre-CTLs require two signals. One of the signal required is class I MHC-antigen complex on a target cell, and the other is provided by cytokines (IL-2, IL-6 and IFN-γ) secreted by activated helper T-cell. The activated pre-CTLs differentiate into mature CTLs, when recognized its target cell, with class I MHC and corresponding antigen, secretes perforin and toxin. Perforin drills holes on target cell and cause cell lysis, and toxin such as lymphotoxin causes apoptosis of target cell. The lymphokines released by helper T-cell not only regulate T- and B-cell but also mobilise other cells of the defence mechanism such as macrophage, killer cell , NK cell, granulocytes etc..

1.4 EFFECTOR MECHANISMS IN ANTI-TUMOR IMMUNITY

Our body defence system does process effectors that can destroy tumor cells named the immunosurveilance hypothesis. This idea was first articulated by Macfarlane Burnet (1970). The effectors may be protective even after malignant cells have grown into tumors. The effectors include; B-cells, killer cells, helper T cells, cytotoxic T cells, natural killer cells and macrophages. However, the hypothesis is challenged by the fact that it is not generally valid for most forms of cancer. For example, if the immune system is required to prevent the frequent occurrence of cancers, one would expect that many more

malignant tumors would develop in individuals with congenital or acquired immuno-deficiencies than in immunocompetent individuals. In fact, this is not the case for most common forms of cancers, such as carcinomas of the colon, lung, or breast. However, there is a remarkably increased incidence of certain forms of cancer in immunosuppressed individuals.

1.4.1 B-CELL

B-cell, when activated by tumor antigens, produces antibody direct against the tumor cells. However, both experimental models studies in human demonstrate that antibody is not very effective in causing tumor rejection, particularly solid tumors. Antibody may play some roles in the control of leukemia. In the rejection of tumor, antibody calls for killer cells and complement to complete the killing (Abbas, 1991). Killer (K) cells (non-T, non-B, null cells) have Fc receptor on their membrane. If they encounter a tumor cell that has IgG molecules on its surface they will interact with that "sensitised" cell and destroy it. The process is called the antibody-dependent cellular cytotoxicity (ADCC). The K cell killing process also involves antibody but requires much less antibody than does ADCC. When complement killing is involved IgM antibody is required. As antibody binds to the surface of the tumor cell, the classical complement pathway is triggered, leading to the eventual destruction of the tumor cell.

1.4.2 NATURAL KILLER (NK) CELLS (NON-T, NON-B)

NK cells are morphologically associated with large granular lymphocytes. It is believed that NK cells are responsible for immunosurveilance. The cells recognise and kill tumor cells via a direct interaction between the NK cells and the tumor cells (by the same

mechanism as CTL cells). NK cells also have Fc receptors; however, these cells can kill tumor cells without any attached antibody (Abbas, 1991).

1.4.3 MACROPHAGES

Activated macrophages are cytotoxic to tumor cells (Filder, 1974 ; Liotta, 1977). The activation can be achieved by several routes. Tumor cells coated with antibodies can activate macrophage via the Fc receptor of macrophage. Lymphokine called macrophage-activating factor (MAF) released from activated T helper cells can also activate macrophage. MAF changes macrophage metabolism, and makes them potent killers of tumor cells. Activated macrophages do not rely on interacting with any specific tumor antigen, but, like NK cells, do seem to distinguish malignant from normal cells. There are probably several mechanisms of macrophage killing of tumor target cells, some of which are essentially the same as the mechanisms of macrophage killing of infectious organisms (Abbas, 1991). These include the release of lysozymal enzymes and reactive oxygen metabolites. Other reactive chemical species, such as nitric oxide, may also play a role. Activated macrophages also secrete cytokines, interferon (IFN) and tumor necrosis factor (TNF), that contribute to tumor cell destruction. Interferons may serve primarily as anti-proliferative agents as well as the activation of NK cells. In addition, interferons augment the expression of class I MHC molecules which make tumors more susceptible to control by immune effector mechanisms. TNF kills tumors by at least two different mechanisms. First, binding of TNF to high-affinity cell surface receptors is directly toxic to tumor cells. The toxicity may be a result of the production of free radicals. Direct toxic effects of TNF may also involve disruption of cytoskeletons. Second, *in vivo* , TNF causes tumor necrosis by impairing the vascularisation of the tumor and interrupt nutrient supply.

1.4.4 CYTOTOXIC T-LYMPHOCYTES (CTLs)

CTLs were demonstrated to be effective anti-tumor effector *in vivo*. The CTLs involved are predominantly class I MHC restricted. These cells may only play role in immunosurveillance of virally induced tumors. Mononuclear cells derived from the inflammatory infiltrate in human solid tumors, called tumor-infiltrating lymphocytes (TILs) were found containing CTLs with the capacity to lyse the tumor from which they were derived (Rosenberg, 1986). However, the specificity of the anti-tumor CTLs derived from peripheral blood or tumors is not well established, since they also show reactivity against unrelated tumor cells. The killing mechanisms of CTLs involve direct cell contact between CTLs and target tumors. After contact, the CTLs release proteins which drills holes on target cell and cause lysis. Fig. 1.1 summarise the effector mechanisms in anti-tumor immunity.

1.5 CANCER TREATMENT

Cancer therapeutic strategies involve surgery, radiotherapy, drug therapy, gene therapy or immunotherapy. The most commonly used therapeutic strategies were limited to surgery, radiotherapy and drug therapy. Gene therapy and immunotherapy are still in their infant stage for practical purposes.

1.5.1 SURGERY

Surgery cures more patients of cancer than do both radiotherapy and cytotoxic chemotherapy together. Surgery is also novel by its minimal side effect. However, surgery do suffer many disadvantages. In case of irreparable organs such as brain and nervous tissues are involved , the use of surgery will be limited. Surgery also fails to cure when

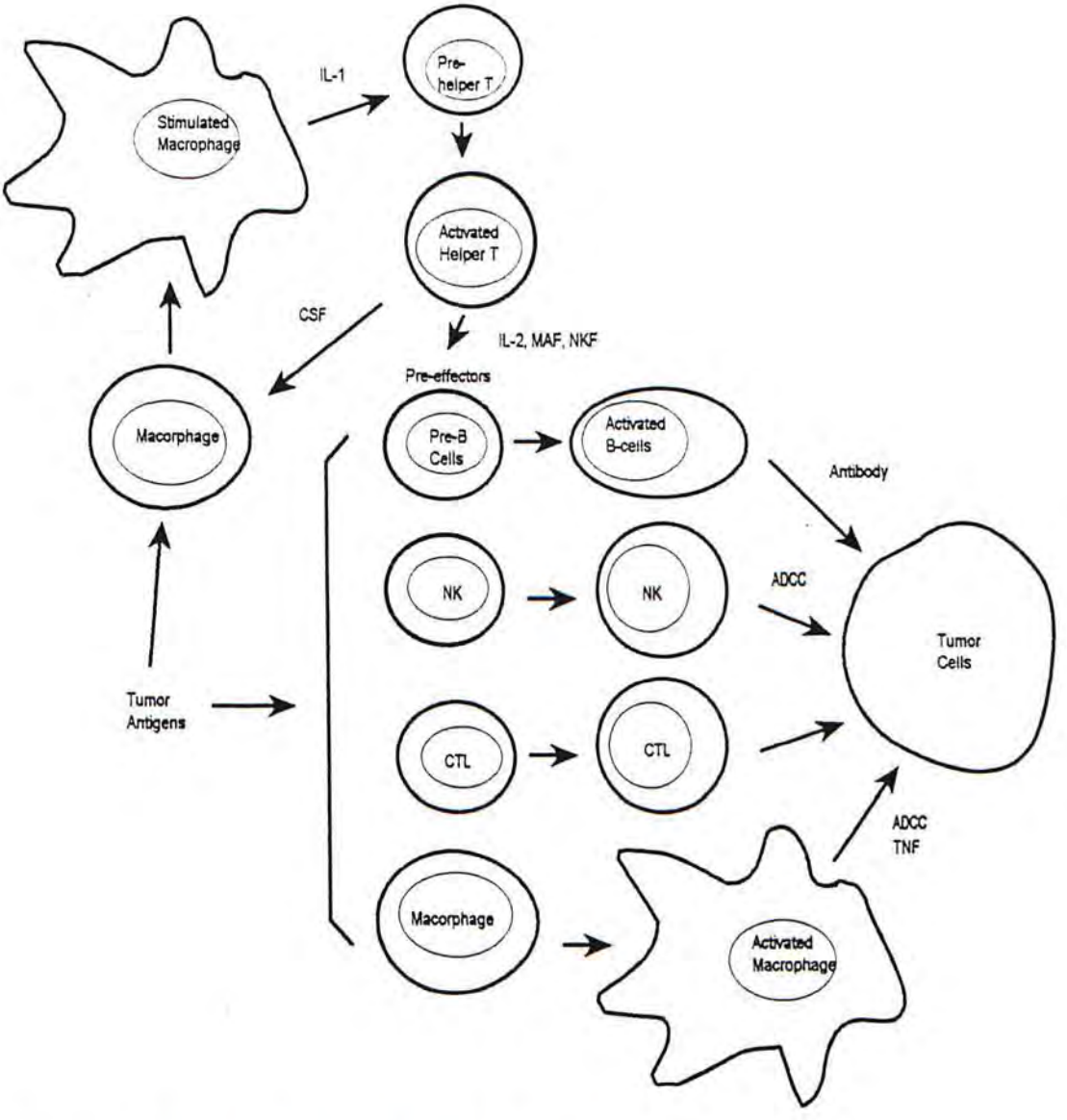


Fig. 1.1 The effector mechanisms in anti-tumor immunity.
ADCC—antibody dependent cell cytotoxicity
CSF—colony stimulating factor
CTL—cytolytic T-lymphocyte
IL-1—interleukin 1
IL-2—interleukin 2
MAF—macrophage activating factor
NK—natural killer cell
TNF—tumor necrosis factor

there is extensive metastasis, or when local excision is incomplete. Furthermore, surgery is not practical for patients of advanced age or poor general health such as patients with anemia will have increased anesthetic risks.

1.5.2 RADIOTHERAPY

Radiotherapy uses ionising radiations to cure cancer. The radiations may be X-ray from a X-ray machine, γ-rays from radionuclides such as ^{60}Co, β-rays or electrons from radionuclides or machine and fast neutrons from cyclotron. The ionising radiations kill cancer cells by converting oxygen to toxic radicals which cause DNA damage and prevent mitosis. A potential advantage of radiotherapy over surgery is that a greater volume of tissue can be treated, but the treated tumor cells are not inevitably eradicated. The ineffective killing may be due to the present of hypoxia within some tumors. Other than these defects, radiotherapy causes severe tissue damage such as dermatitis, leucocytopenia etc..

1.5.3 DRUG THERAPY

In routine clinical practices, anti-cancer drugs fall into two main groups, hormonal and cytotoxic. Hormonal agents are generally much better tolerated than cytotoxic drugs which can cause severe morbidity and even death. Other than toxicity, cytotoxic drugs usually induce resistance (Hayes and Wolf, 1990). The curative potential of these drugs are largely confined to relatively uncommon tumors.

1.5.4 GENE THERAPY

The development of gene therapy and immunotherapy aims to kill cancer selectively in order to minimise the disadvantage of surgery, radiotherapy and drug therapy. One example of gene therapy, virus-directed enzyme/prodrug therapy (Huber, 1991), involves the infection of hepatoma with retrovirus bearing varicella-zoster virus thymidine kinase (VZV TK) gene. The gene is expressed more effectively in neoplastic cells than normal liver cells. Expressed VZV TK product can activate a nontoxic prodrug , 6-methoxypurine arabinonucleoside (araM) to cytotoxic anabolite adenine arabinonucleoside triphosphate (ara ATP) and thus achieve specific killing.

1.5.5 IMMUNOTHERAPY

Immunotherapy, which includes antibody therapy, adoptive cellular immunotherapy, cytokine therapy and stimulation of immune effectors, takes advantage of the natural effector of the defence mechanism. Antibody therapy treats cancer by raising antibody against specific tumor antigens. The antibody with or without conjugate (drug, toxin, radioisotope etc.) is used to kill cancer specifically (Pastan, 1991). In adoptive cellular immunotherapy, cells such as natural killer cells and tumor-infiltrating lymphocytes (Rosenberg, 1986) with tumor killing capacity, were isolated from patients and cultured *in vitro* with IL-2. After culture, activated cells may be administered either together with or without IL-2 into the patients for curative purpose (Rosenberg, 1988,1989).

Other than contribution to adoptive cellular immunotherapy, cytokines when used alone also have curative potential. Cytokines such as IL-2, IL-4, TNF, IFN-α, IFN-γ, GM-CSF and G-CSF are currently under clinical trials (Jernberg-Wiklund, 1991). The use of these cytokines aims to activate the *in vivo* immune effectors to kill cancer. Although

these cytokines are present endogenously, when high dosage is administered (dosage usually required to give effective treatment), severe side effects may occur.

Another way to stimulate the immune effectors to kill cancer cells is achieved by exogenous products such as BCG, glucan, Levamisole (Proctor *et al*, 1977) etc.. The products have great variations in nature. These products also derived from diversed sources such as tumor cell antigen (from tumor cells), lipids, proteins or polysaccharides (from higher plants, algae, fungi and bacteria) and Levamisole from synthetic compounds. These products stimulate the non-specific endogenous immune responses.

1.6 NON-CYTOTOXIC ANTITUMOR POLYSACCHARIDES OF FUNGI

Botanical source (higher plants, algae, fungi and bacteria) is the major source of non-cytotoxic antitumor polysaccharides (Whistler, 1976). These polysaccharides varied a lot in their components and linkages. The polysaccharides may contain phosphate group, amino group, O-acetylated group, uronic acids, amino acids and the mono sugar units—glucose, mannose, galactose, arabinose, xylose etc.. The sugar units make up the polysaccharide skeleton may be linked together by α-, β-, (1→2), (1→3), (1→4) or (1→6)-linkages (Whistler, 1976). Even there exist many variables that contribute to the great diversity of the polysaccharides, the non-cytotoxic antitumor polysaccharides of fungi are quite unique in their compositions and structure. The antitumor fungal polysaccharides was roughly divided into three major groups; yeast polysaccharides, lichen polysaccharides and fungal polysaccharides (include all fungi except yeast and lichen).

1.6.1 YEAST POLYSACCHARIDES

Zymosan, composed of essentially intact yeast cell wall roughly contains equal amount of β-(1→3)-D-glucan and α-mannan (Bacon *et al*, 1969), has been reported to be

active against solid tumors such as Sarcoma 37, and Sarcoma 180 in ICR albino mice (Bradner *et al*, 1958), whereas ascites tumor were not responsive. Mannan fractions are obtained from the hot-water extract of yeast cells, and glucan fractions are obtained by alkaline extraction of the residue. Depending on the species and the method of fractionation, mannan or glucomannan, or both can be obtained. It was found that glucan, mannan and glucomannan extracted were all active. However, mannans are more active than the glucan fraction. The activity of the glucomannan fraction seems to depend on the content of D-glucose and, perhaps, the linkages. Their antitumor activities and compositions are given in Table 1.1.

1.6.2 LICHEN POLYSACCHARIDES

Various glucans, such as pustulan, lichenan, and isolichenan, isolated from lichens, have been found to be active against solid sarcomas in mice. Pustulan-like glucans containing β-(1→6)-D-linkages have been isolated from *Gyrophera esculenta* and *Lassalia papulose* (Shibata *et al*, 1968), but these glucans differed from pustulan in containing 4-10 % of acetyl groups situated at O-3. Deacetylation lowered the activity, and replacement of O-acetyl groups by O-methyl groups, or complete acetylation of the glucan , yielded inactive product (Whistler , 1965). Similar, partially acetylated glucans have been isolated from *Umbilicaria* sp., and lichenan [a β-(1→3)-, (1→4)-D glucan] and isolichenan [an α-(1→3)-, (1→4)-D-glucan] have been isolated from *Cetraria* sp.. An antitumor lichenan has also been isolated from *Alectoria* sp. Other lichens that have yielded antitumor glucans are *Parmelia caperata, Clandomia mitus, Usnea baylei* and *Evernia prunastri* (Whistler, 1976). These glucans were active against Sarcoma 180 solid tumors, and inactive against ascites tumors. The antitumor activity, compositions and structure of various glucans isolated from lichens are given in Table 1.2.

Antitumor Activity of Yeast Polysaccharide Against Subcutaneously Implanted Sarcoma 180 in Mice

Polysaccharide	Yeast	Composition	Dose(mg/kg ×number)	Route	Regression	Inhibition Ratio (%)
Glucan	*S. cerevisiae*	83% Glc	150 × 10	i.p.	4/9	88.6
Mannan	*S. cerevisiae*	99% Man	150 × 10, 100 × 10	i.p.	7/10, 7/8	95.9, 100.0
Mannan	*C. albicans*	99% Man	200 × 1, 150 × 9	i.p.	4/9	64.8
Glucomannan	*C. albicans*	63% Man, 37% Glc	200 × 1, 150 × 9	i.p.	6/8	99.1
Glucomannan	*C. utilis*	56.5% Man, 43.5% Glc	200 × 10	i.p.	0/7	9.1
Glucomannan	*C. utilis*	92% Man, 8% Glc	100 × 10, 100 × 10	i.p., i.v.	8/10, 3/10	98.8, 100.0
O-(Carboxymethyl)-mannan	*C. utilis*	92% Man, 8% Glc	100 × 10, 100 × 10	i.p., i.v.	5/10, 7/10	91.7, 98.2

Polysaccharides were administered i.p. or i.v. in the appropriate dose, starting 24 h after tumor implantation, and the results were recorded after 5 weeks. (Data were extracted from Roy L. Whistler, 1976)

TABLE 1.1

Antitumor Activity of Lichen Glucans Against Subcutaneously Implanted Sarcoma 180 in Mice

Glucan	Source	Linkages	Dose (mg/ kg)	Regression	Inhibition Ratio (%)
Pustulan	*Gyrophera esculenta*	β-(1→6)-D	150	1/8	85.5
Lichenan	*Cetraria islandica*	β-(1→3)-,(1→4)-D	200, 150, 100	5/6, 8/8, 8/10	99.1, 100, 99.7
Lichenan	*Evernia prunastri*	β-(1→3)-,(1→4)-D	200	10/10	100
Isolichenan	*Cetraria islandica*	α-(1→3)-,(1→4)-D	200, 150	6/8, 7/8	99.6, 98.9

Glucans were administered i.p., daily for ten days, starting 24 h after tumor implantation, and the results were recorded after 5 weeks. (Data were extracted from Roy L. Whistler, 1976)

TABLE 1.2

1.6.3 FUNGAL POLYSACCHARIDES

Crude, fungal extracts from a number of fungi (ascomycetes, basidiomycetes, deuteromycetes and oomycetes) have been reported to be active against experimental tumors, and, in some cases, they showed definite palliative action on non-operable, human tumors (Whistler, 1976). A number of antitumor polysaccharides have been extracted from the fungi. The skeleton of the extracted polysaccharides composed mainly of β-(1→3)-linked D-glucose and small proportions of D-galactose and D-mannose have been extracted from fruit bodies, mycelia, and from culture fluids of various fungi such as *Cereosparie cryptomeriae*, *Chaetomiun cochlides*, *Cladosporium fulvum*, *Cochliobolus miyabeanus* (Nanba and Kuroda,1987), *Grifola frondosa* (Ohno *et al*, 1984,1986), *Lentinus edodes* (Chihara *et al*, 1970 and Togami *et al*, 1982), *Pholiota namenko*, *Phytophthora* species (Bruneteau *et al*, 1988), *Pleurotus ostreatus* (Yoshioka *et al*, 1972), *Poria cocos*, *Pyrensphora teres*, *Sclerotinia sclerotiorum* etc.. Such polysaccharides have shown activity against solid tumors such as sarcoma 37 and sarcoma 180, whereas no response was seen against ascites sarcoma and syngeneic tumors. Antitumor polysaccharides with skeleton deviate from the linear β- (1→3)-linked D-glucose structure have also been extracted from some fungi.

Polysaccharides from *Tremella* differ from other fungal polysaccharides in chemical composition, being composed of D-xylose, D-mannose, and D-glucuronic acid. Other variations includes β-(1→4)-linked D-glucans from *Lentinus edodes* (Sasaki *et al*, 1976), β-(1→3)- and (1→4)-linked D-glucans from *Ganoderma applanatum* (Sasaki *et al*, 1971) and *Phellinus linteus* (Sasaki *et al*, 1971), a β-(1→3)-D-glucan having a β-D-glucopyranosyl group linked (1→6) to every third to fourth residue of the main chain from *Schizophyllum commune* (Yamamoto *et al*, 1981) etc.. Variations, due to frequency and length of branch, also exist. Nevertheless, they all show activity. Their antitumor activity, compositions and structure are shown in Table 1.3.

Antitumor Activity of Some Fungal Polysaccharides Against Subcutaneously Implanted Sarcoma 180 in Mice

Glucan	Source	Linkages	Dose (mg /kg× number)	Route	Regression	Inhibition Ratio (%)
Lentinan	*Lentinus edodes*	β-(1→3)-D	25 × 10, 5 × 10, 1 × 10, 0.2 × 10	i.p., i.p., i.p., i.p.	2/9, 7/10, 6/10, 6/10	73.0, 97.5, 95.1, 78.1
Schizophyllan	*Schizophyllum commune*	β-(1→3)-, (1→6)-D	5 × 10, 1 × 10, 0.5 × 10, 5 × 4, 1× 4, 10× 10, 1 × 10	i.p., i.p., i.p. i.v., i.v. s.c., s.c.	4/10, 7/10, 7/10, 5/10, 4/10, 4/10, 0/10	89.0, 81.0, 82.0, 100.0, 96.0, 82.0, 11.0
Pachymaran	pachyman from *Poria cocos*	β-(1→3)-D	5 × 10	i.p.	4/9	96.0
Scleroglucan	*Sclerotium glucanicum*	β-(1→3)-, (1→6)-D	50 × 10, 5 × 10, 0.5 × 10	i.p., i.p., i.p.	2/10, 5/10, 7/10	41.2, 88.2, 91.6
Fraction LC-I	*Lentinus edodes*	β-(1→4)-, (1→6)-D	30 × 10, 15 × 10, 5 × 10	i.p., i.p., i.p.	8/9, 10/10, 8/10	96.5, 100.0, 99.0

Treatment with glucans was started 24 h after tumor implantation, and the results were recorded after 5 weeks. (Data were extracted from Roy L. Whistler, 1976)

TABLE 1.3

1.7 FUNGI AND THEIR POLYSACCHARIDES

Fungi can simply be defined as walled eukaryotic organisms whose nutrition depends on absorption of preformed organic matter by passive diffusion. Kingdom of fungi was roughly divided into five major groups; Oomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes, according to their structure, physiology and biochemistry (Bartnicki-Garcia, 1961).

The polysaccharides produced by fungi are quite diverse (Lewis, 1991). The different form of polysaccharides may act as either reserve carbohydrates or structural carbohydrates. Fungal polysaccharides are structurally quite different from polysaccharides of other natural sources, and they are grouped according to their component sugars and predominant configurations, or in the case of heteropolymers, the structure of the main chain.

1.7.1 RESERVE CARBOHYDRATES

Reserve carbohydrates in fungi can be found intracellularly (cytoplasmic) or extracellularly (e.g. culture medium). All the reserve carbohydrates act as only carbon reserve and energy reserve except polyols (Smith and Berry, 1978); the commonest and most abundant soluble carbohydrates in mycelia of most fungi (Lewis and Smith, 1967). Polyols are sugar alcohol such as mannitol and arabitol. Trehalose is an α, α-diglucoside present in cytoplasm. Other less common carbohydrate reserves include glycogen, starch, mycodextran (nigeran), α-D-glucan, β-D-glucan, polygalactose, and acidic polysaccharides. Glycogen and starch present in fungi intracellularly. They, both have similar structures, consist of multiple branched molecules containing numerous chains of α-(1→4) - linked D-glucose residues except starch possesses additional linear α-(1→4) - linked D-glucose polymers. Mycodextran, exists intracellularly, which is a linear polymer

of D-glucose with alternate α-(1→3)-D- and α-(1→4)-D- linked glucopyranosyl residues (Gorin & Spencer, 1968). α-D-glucan such as pullulan produced by *Pullularia pullulans* present extracellularly. α-D-glucan differ from mycodextran by its linkage. Unlike mycodextran, α-D-glucan such as pseudonigeran, derived from *Aspergillus niger* NRRL326, contains predominantly α-(1→3)-D- linkages (87%) and some α-(1→4) -D- linkages (13%) instead of alternate α-(1→3) -D- and α-(1→4)-D-linkages only. β-D-Glucans serve as reserve are present intracellularly and chiefly contains 1→3 linkages. It may also contain branched β-D-glucans with 1→4 linkage and 1→6 linkage. Linear β-D-glucan with exclusively (1→6) linkage also exist (Manners *et al*, 1974). Freeman and Macpherson (1949) described two extracellular polysaccharides produced by strains of *Penicillium luteum*, a neutral polygalactose and an acidic polyglucose. Other extracellular acidic polysaccharides such as malanogalactan and peptidophosphogalacto-mannans were also reported (Gander, 1974).

1.7.2 STRUCTURAL POLYSACCHARIDES

Structural polysaccharides of fungi are mainly restricted to the cell wall . Unlike plant cell wall , fungal cell wall has great variations in its major wall components (Smith and Berry, 1978). For example, the major components can be chitin, cellulose, β-D-glucan, glycogen, chitosan, mannan, galactosamine and galactose. Chitin is the commonest major wall polysaccharides of fungi. It is a linear polymer of N-acetyl glucosamine linked by β-(1→4) glycosidic bond. Cellulose, the major wall component of plant, exists as major wall polysaccharides of certain fungal group. It is a linear polymer of glucose linked by β-(1→4) glycosidic bond. β-D-glucans are glucose homopolymer with β-(1→3)-linkage (unbranched). Variations due to the presence of β-(1→ 4) or β-(1→6) linkages also exist. Glycogen, a reserve polysaccharide, does contribute to the wall structure of certain fungi (Alexopoulos and Mims, 1979). It is a multiply branched molecules containing many

chains of α-(1→4)-linked glucose. Chitosan is a linear polymer of glucosamine linked by β -(1→4)-linkage. Mannan, is a homopolymer of mannose, which may contain α-(1→2)-D-, α-(1→3)-D-, α-(1→6)-D-, β-(1→3)-D- and β-(1→4)-D-linkages. Polysaccharides, such as α-D-glucans with α-(1→3)- and α-(1→4)-linkage, heteropolysaccharides and acidic polysaccharides also contribute to the wall structure but restricted to matrix components but not skeletal elements. All fungi can be divided into three groups according to their major skeletal elements; the chitinous group, the cellulosic group and the miscellaneous group (O'Brien and Ralph, 1966). Table 1.4 shows representative fungal member of different cell wall category.

1.8 THE ARCHITECTURE OF THE FUNGAL CELL WALL

In this paragraph, only the architecture of chitinous group will be described as the target fungus-*Flammulina velutipes*-of my project , belongs to this group.

The wall of chitinous fungi adapt a microfibrillar structure (Wessels *et al*, 1989): a skeleton of interwoven microfibrils, conferring tensile strength, embedded in an amorphous matrix. The microfibril is a group of parallel unsubstituted alkali-insoluble glycosaminoglycan (chitin) chains associated by hydrogen-bonding to form fibril structure. The microfibrils form a stiff network of interconnected microfibrils by hydrogen-bond of substituted glycosaminoglycan chains between chitin microfibrils. Three chains of β-(1→ 3)-D-glucans twisted to form a triple helices. The hydroxyl group of the terminal glucose residues of the helices will condense (by Schiff reaction) with the amino group of the glucosaminoglycan to form a covalent attachment (Stagg and Feather, 1973). This further strengthen the microfibril network structure. Some β-D-glucoses may remain unattached in mature cell wall and are embedded in the amorphous matrix which fill up the spaces

between the network. Fig. 1.2 illustrates the arrangement of the wall components. Other than polysaccharides, the amorphous matrix do contain a few proteins and lipids.

Cell Wall Composition in the Fungi. (Modified slightly from Barnicki-Garcia, 1970)

Fungal Group	Cell Wall Category	Representative Genera
Chitinous	Chitin-Chitosan	*Mucor, Phycomyces, Zygorhynchus*
	Chitin-β-Glucan	*Allomyces, Neurospora, Aspergillus*
	Chitin-Mannan	*Sporobolomyces, Rhodotorula*
Cellulosic	Cellulose-Glycogen	*Polysphondylium, Dictyostelium*
	Cellulose-β-Glucan	*Phytophthora, Pythium, Saprolegnia*
	Cellulose-Chitin	*Rhizidiomyces*
Miscellaneous	Mannan-β-Glucan	*Saccharomyces, Candida*
	Galactosamine-Galactose polymers	*Amoebidium*

TABLE 1.4

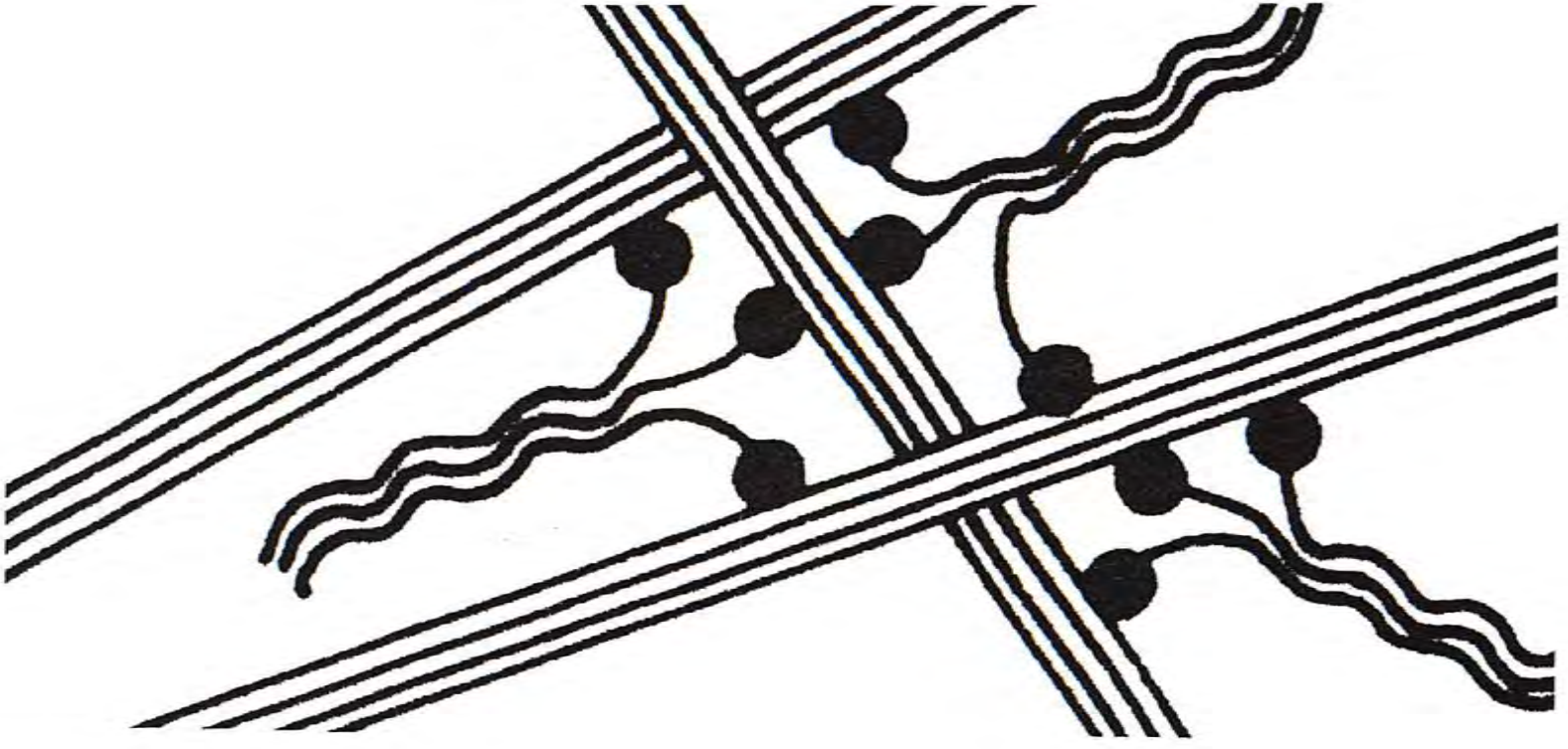


Fig. 1.2 The figure shows the interactions between glucosaminoglycan (chitin) chains (*straight lines*) β-(1→3)-D-glucan (*wavy lines*). Hydrogen-bonding between unsubstituted glucosaminoglycan chains may produce chitin microfibrils. Triple helices formed by the bound glucan chains interconnect glucosaminoglycan chains (The picture was extracted from Wessels, 1990).

Materials and Methods

CHAPTER TWO
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 ANIMALS

Inbred female BALB/c, female C57BL/6J and outbred male ICR albino mice were bred at the Animal House of the Chinese University of Hong Kong. They were fed by animal diet (Chow 5001, Rodent Lab.). Except as indicated, female BALB/c and female C57BL/6J mice at the age of 6-8 week old and male ICR albino mice weighted at 38 g were used in each experiment.

2.1.2 MUSHROOMS

Fresh fruiting bodies of *Flammulina velutipes*, *Lyophyllum aggregatum* (*shimeji*) and *Volvariella volvacea* were brought from Yaohan, a local department store. The mushrooms were imported from Japan.

2.1.3 BUFFERS, CULTURE MEDIA AND CHEMICALS

Phosphate-buffered-saline

PBS was prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4.2H_2O$, 0.2 g KH_2PO_4 in 1 litre double distilled water (ddH_2O). The pH of the solution was adjusted to 7.4 with 1 M NaOH or 1 M HCl. The solution was sterilised by autoclaving at 121 ^oC for 20 min.

Hank's Balanced Salt Solution (HBSS)

Hank's Balanced Salt powder (Sigma Chem. Co., U.S.A.) for 1 litre preparation was dissolved in 1 litre of ddH_2O containing 0.35 g sodium bicarbonate. The solution was adjusted to pH 7.4 and sterilised by membrane filtration.

RPMI-1640 Medium

RPMI-1640 powder (Gibco Co., U.S.A.) for 1 litre preparation was dissolved in 1 litre of ddH_2O containing 0.85 g $NaHCO_3$. The solution was adjusted to pH 7.4 and sterilised by membrane filtration. RPMI medium without supplement other than 1 % antibiotics was usually used in washing cells and was designated as "plain RPMI medium".

Serum Supplements

Fetal calf serum (FCS) was obtained from Flow Lab., Australia and Gibco, U.S.A. FCS was prepared as 50 ml aliquots and stored at -20 ^{o}C until use. Heat-inactivated FCS (HIFCS) was prepared by incubating the aliquots at 56 ^{o}C for 30 min. FCS was usually used as a supplement to growth medium for cell culture *in vitro* and the final concentration was normally 10 %. HIFCS, on the other hand , was used as a serum supplement for cytotoxicity and cytostatic assays.

Penicillin-Streptomycin-Fungizone Solution (PSF)

PSF was brought from Gibco, U.S.A. Stock solution (100×) containing 10,000 units/ml penicillin G, 10,000 μg/ml of stretomycin sulfate and 300 μg/ml of amphotericin B was stored at -20 ^{o}C as 5 ml aliquots. Usually, 5 ml PSF stock was added to 500 ml RPMI medium containing 10% FCS as the "complete medium" for *in vitro* cell culture.

Antibodies

Monoclonal rat anti-mouse Thy 1.2 antibody (clone F7D5) was purchased from Serotec Co., U.K. and used at a dilution of 1:1,000 in RPMI medium.

Rabbit anti-sheep red blood cell antibody (SRBC) was purchased from (Accurate Chemicals and Scientific Co., U.S.A.) and was stored at -20 ^{o}C as 250 µl aliquots.

Barley-β-D-glucan

This is a soluble β-(1→3)-D-glucan derived from barley and was purchased from Sigma Chem. Co., U.S.A.. The glucan was dissolved in ddH_2O (3 mg/ml) and stored at -20 ^{o}C as 1 ml aliquots.

β-(1→3)-D-glucan

This is an insoluble β-(1→3)-D-glucan derived from *Pleurotus ostreatus* and was purchased from Sigma Chem. Co., U.S.A.. The glucan was suspended in ddH_2O (3 mg/ml) and stored at -20 ^{o}C as 1 ml aliquots.

Complement (C')

Hemo-Lo guinea pig complement was purchased from Cedarlane Lab., Canada. The lyophylised powder was reconstituted with ice cold distilled water, distributed in 1 ml aliquots and immediately frozen at -70 ^{o}C until use.

Laminarin

This is a soluble β-(1→3)-D-glucan derived from a sea brown algae, *Laminaria digitata*, and was purchased from Sigma Chem. Co., U.S.A.. The glucan was suspended in ddH_2O (3 mg/ml) and stored at -20 ^{o}C as 1 ml aliquots.

Laminarinase

This is a 1→3-(1→3;1→4)-β-D-Glucan 3(4)-glucanohydrolase, whose natural substrate is Laminarin. This was purchased from Sigma Chem. Co., U.S.A.

Plant gelatin

Type V iota-carrageenan, derived from *Gigartina aciculaire* and *Gigartina pistillata*, was purchased from Sigma Chem. Co., U.S.A.. The plant gelatin was used as a standard in periodate oxidation and to prepare Gelatin veronal buffer (GVB).

Pyridine

This organic solvent was purchased from E. Merck, Germany and was used as developing solvent in chromatographic separation.

Schiff's reagent

The reagent was purchased from BDH, England. The reagent was used to stain ingested yeast cell wall particle in phagocytic assay.

Chromatographic materials

1. B-cell depleting column kit

The B-cell depleting kit was purchased from Cedarlane Lab. Ltd., Canada. The column was used to prepare T-cell population from splenocytes.

2. TLC plate

The glass plate is precoated with Kiesegel 60 F_{254} of dimensions: 20 cm× 20 cm. The plates were purchased from E. Merck, Germany.

3. Sephadex G-100 and G-200

The gel was purchased from Pharmicia LKB, Sweden and was used to estimate the molecular weight of the separated polysaccharides.

Dyes

1. Aniline Blue

This is purchased from BDH England. This dye was used to screen potential mushroom which has β-(1→3)-D-glucan in its cell wall.

2. Congo Red Solution

3.8 μM Congo red (Sigma Chem. Co., U.S.A.) in ddH_2O was used to identify β-(1→3)-D-glucan.

3. Neutral Red Solution

0.5% (w/v) neutral red (Sigma Chem. Co., U.S.A.) in PBS was used as a vital dye for the staining of viable cells.

4. MTT dye

MTT dye (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma Chem. Co., U.S.A.. The dye was used in colorimetric cytotoxicity assay.

5. Protein Assay Dye Reagent

The dye is purchased from Bio-Rad U.S.A. and was used to determine the protein content of the separated polysaccharide fractions.

6. Tryphan Blue Solution

0.1% (w/v) tryphan blue (Sigma Chem. Co., U.S.A.) in PBS was used to assess the viability of cells.

Elisa Kits

1. Murine GM-CSF Elisa Kit

The kit was purchased from Endogen, U.S.A.. The kit was used to detect serum GM-CSF level of experimenting mice and the use of the kit strictly followed the instructions of the kit.

2. Murine Interferon-γ Elisa Kit

The kit was purchased from Genzyme, U.S.A.. The kit was used to detect serum Interferon-γ level of experimenting mice and the use of the kit strictly followed the instructions of the kit.

3. Murine Interleukin-1 α Elisa Kit

The kit was purchased from Genzyme, U.S.A.. The kit was used to detect serum IL-1 α level of experimenting mice and the use of the kit strictly followed the instructions of the kit.

4. Murine Interleukin-4 Elisa Kit

The kit was purchased from Endogen, U.S.A.. The kit was used to detect serum IL-4 level of experimenting mice and the use of the kit strictly followed the instructions of the kit.

Ficoll-Paque Solution

The sterile solution was purchased from Pharmacia LKB, Sweden. This is used for *in vitro* lymphocyte isolation.

Mitogens

Mitogens such as concanavalin A (ConA) and lipopolysaccharide (LPS) were purchased from the Sigma Chem. Co., U.S.A.. They were dissolved in PBS (1 mg/ml), filtered with 0.45 μm millipore membrane and stored as 300 μl aliquots at -20 ^{o}C until use.

Scintillant

Scintillant was prepared by mixing 0.01% (w/v) dimethyl-1,4-bis(2-(5-phenyloxazol)) benzene (POPOP) and 0.4% (w/v) 2,5-diphenyloxazole (PPO) in toluene (Fisher Chem. Co., U.S.A.) and was stirred overnight before use.

Sheep Red Blood Cells (SRBC)

Sheep red blood cells (SRBC) were purchased from Serotec Co., U.K..

(Methyl-^{3}H) Thymidine (^{3}H-TdR) Solution

(Methyl-^{3}H) Thymidine (1 mCi/ml) was purchased from Amersham, U.K.. 10 μ Ci/ml working solution was prepared by diluting the stock solution with complete RPMI medium. For pulse labeling, 50 μl working solution was added to each culture well.

Yeast

Yeast (*Saccharomyces cerevisiae*), was purchased from Sigma Chem. Co., U.S.A., was used to prepare yeast cell wall. The yeast cell wall was used in phagocytic and antitumor assay.

2.1.4 Cell lines

(1) Fibroblast-like Tumors (L-929) :

This is a transformed fibroblast cell line derived from C3H mice.

(2) Macrophage-like tumor (PU5-1.8) :

This is a spontaneous macrophage-like tumor derived from BALB/c mice.

(3) Mouse Sarcoma (Sc-180) :

This cell line was derived from Swiss Webster Sarcoma 180 ascites. All these cell lines (with the exception of fibroblast cell lines) were maintained in suspension cultures in complete RPMI medium and sub-cultured every 2 to 3 days. The fibroblast cell lines, on the other hand, were maintained in 75 cm^2 tissue culture flask (Costar, U.S.A.) in 30 ml complete RPMI medium. When the cells nearly formed a confluent monolayer, they were trypsinized with trypsin (0.1%)-EDTA (0.04%) solution (Gibco, U.S.A.) for 2 min. at 37 ^oC, washed three times with complete RPMI medium and 10^5 viable cells were seeded per flask.

2.2 METHODS

2.2.1 SCREENING OF β-(1→3)-D-GLUCAN

Methods of Nakanishi *et al* (1974), with slight modifications, was used. Aniline blue dye (1 mg/ml dH_2O), 0.7 ml, was mixed with 0.7 ml solid sample suspension (100 mg/1.5 ml dH_2O). The mixture was equilibrated for 2 hr. with shaking at room temperature. Solid particles were spun down by centrifugation (10,000×g, 10 min.) and supernatant was removed. The residues were washed twice with methanol and twice with dH_2O. In the washing processes, washing solvent was added to a final volume of 1.5 ml and equilibrated at room temperature for 1 hr., with shaking. After washing, the color of the pellet was noted. A blue color means there exist β-(1→3)-D-glucan in the solid pellet.

2.2.2 EXTRACTION AND FRACTIONATION OF *Flammulina velutipes*

The procedures for the preparation of the FH and SFAI from *Flammulina velutipes* (F.V.) are shown in Fig. 2.1. The method was adopted from Kraus *et al* (1992) with slight modifications. One kg of the fresh fruiting bodies of the mushroom were lyophylised and homogenised with a Waring blender. The dried powder was washed with PBS at 4 °C for 2 hr. The residues were spun down and washed again with PBS at 4 °C for 1 hr. Residues from the PBS washing were washed sequentially with 2× ddH_2O, 2× 1:1 $CHCl_3$-MeOH, 2× acetone and 2× dH_2O (at 20 °C for 30 min. for each wash). The residues from these washing processes were lyophylised and yielded the cell wall materials.

The dried cell wall materials was suspended in dH_2O (1 g/100 ml) and boiled at 121 oC for 30 min. The boiled suspension was filtered through a glass fiber filter GF/C (Whatman, U.S.A.) and the filtered supernatant was lyophylised and designated FH. The

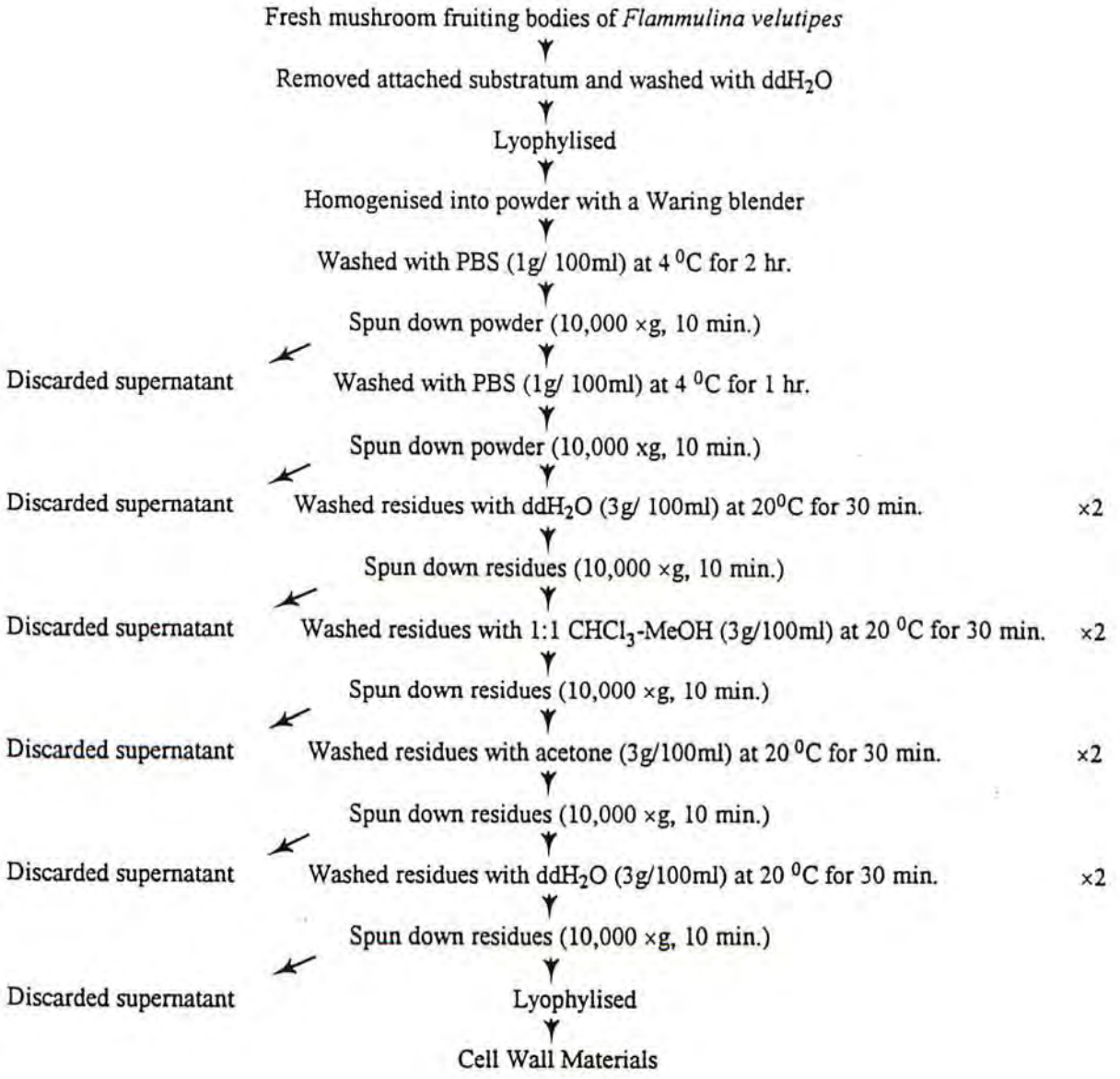


Fig 2.1 A flow chart showing the preparation of the Cell Wall Materials of *Flammulina velutipes*

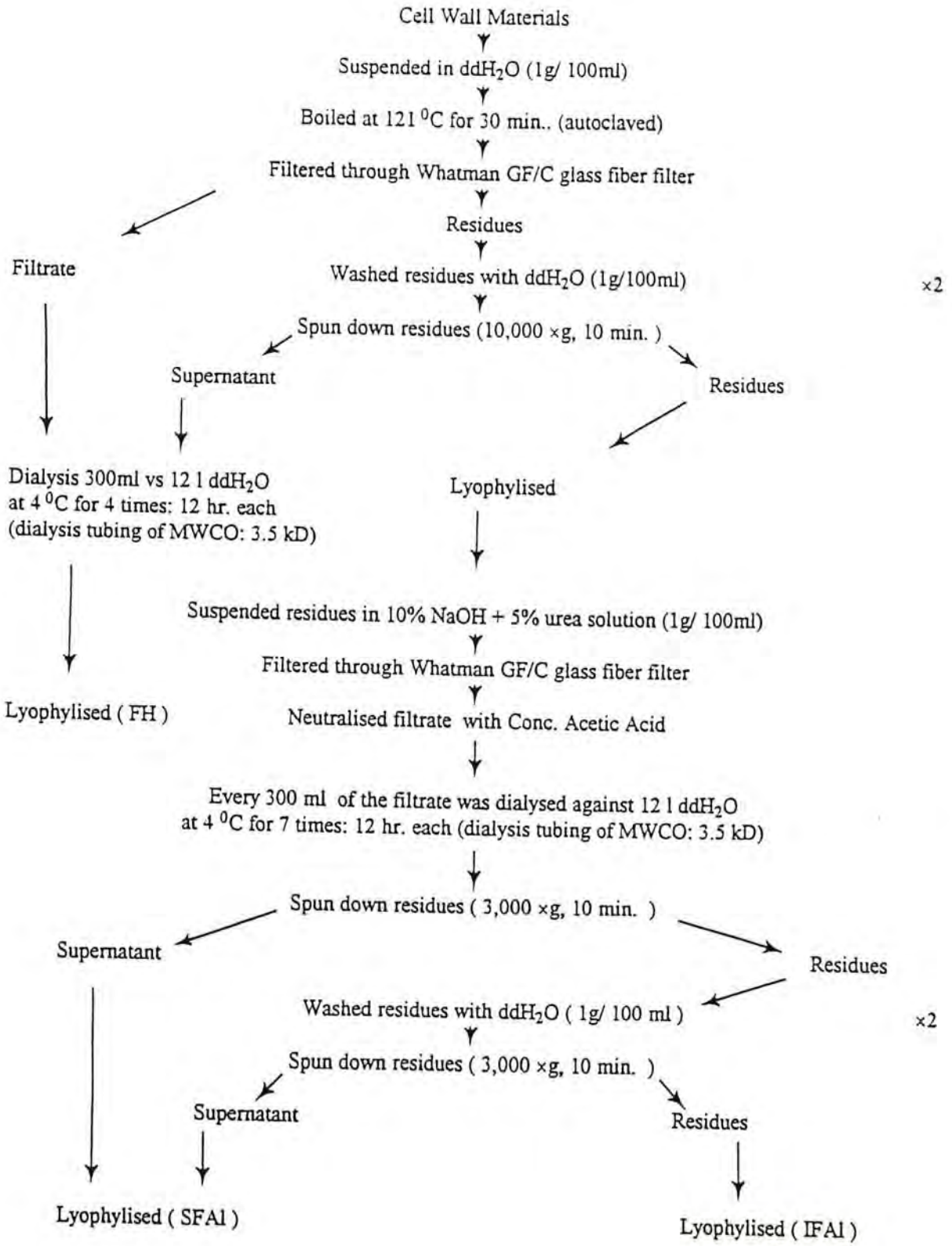


Fig. 2.1 A flow chart showing the preparation of FH, FAl, SFAl and IFAl

residues from the filtration process was washed extensively with ddH_2O to remove any remaining FH and then lyophylised.

The boiled and dried residues was used to prepare the fractions: FAI, SFAI and IFAI. The residues was soaked in 10% NaOH + 5% urea solution (1g /100 ml) at 65 °C for 1.5 hr. (Ohno *et al*, 1986). After treatment, the alkaline solution was filtered through a glass fiber filter GF/C. The filtered supernatant was neutralised with concentrated acetic acid. The neutralised solution, 300 ml, was then dialysed against 7 × 12 litre ddH_2O for 12 hr. each by using dialysis tubing of MWCO: 3.5 kD (Spectrum Medical Industrial, Inc. U.S.A.). The dialysed solution was lyophylised to yield FAI fraction which is a mixture of water soluble and water insoluble polysaccharides. The water insoluble polysaccharide was spun down by a high speed centrifuge (Beckman, Model J2-21, U.S.A.) at 3,000 ×g for 30 min. The supernatant obtained was lyophylised and designated SFAI. The spun down residues was extensively washed with ddH_2O and lyophylised to yield IFAI. In this project, both FH and SFAI were selected as the targets for the investigation of their potential antitumor activity.

2.2.3 CHARACTERISATION OF *Flammulina velutipes*

2.2.3.1 The Determination of Carbohydrate Content of F.V. Fractions

The method of Dubois *et al* (1956) was adopted. In the assay, dextran (MW 72,200) was used as a standard. Phenol reagent (250 µl, 5 %) was mixed with 250 µl sample (1 mg/ml dH_2O). To the 500 µl mixture, 5 ml concentrated sulfuric acid was added and waited for 20 min. at room temperature. The absorbance of the reaction mixture at 490 nm was then measured. The amount of carbohydrate present in FH, FAI, SFAI and IFAI was calculated from the dextran standard curve.

2.2.3.2 The Determination of Protein Content of F.V. Fractions

The Bradford microprotein assay (Bradford, 1976) was used in protein content determination. In the determination, BSA was used as protein standard. 0.8 Ml sample (1 mg/ml dH_2O) was allowed to mix with 0.2 ml, filtered and non-diluted, Bradford reagent (Bio-Rad, U.S.A.). The reaction mixture was allowed to wait for 20 min. before absorbance at 595 nm was measured.

2.2.3.3 The Determination of Uronic Acid Content of F.V. Fractions

The carbazole reaction (Chaplin and Kennedy, 1986) was adpoted. Borate sulfuric-acid reagent (1500 μl) was added to screw-capped tubes and cooled in ice. Standard or test solutions, 250 μl, were carefully layered onto the surface of the borate-sulfuric acid mixture and allowed to diffuse for 10 min. After diffusion, the tubes were gently mixed in ice cool water. When thoroughly mixed, the tubes were heated in a boiling water bath for 10 min., then cooled in ice bath. Cooled carbazole, 50 μl, was added to the mixture and vortexed. The mixture was boiled in a water bath for 15 min. The boiled mixture was cooled in an ice-bath, and the absorbance was read at 525 nm.

2.2.3.4 The Determination of Component Sugar Units of F.V. Fractions

The method of Szystkiewicz and Demetriou (1971) was adopted for chromatographic separation of monosugar units from hydrolysed samples. Hydrolysis was done by adding 1.2 ml FH, SFAl, IFAl, β-(1→3)-D-glucan (*Pleurotus ostreatus*) or glucose (3 mg/ml) to 0.6 ml 12 M HCl. The mixture was injected into a one end sealed Pasteur pipettes with a syringe. The Pasteur pipettes containing the samples were boiled in a 100 °C water bath for 5 min., to drive away air in the Pasteur pipettes. After boiling, the

open end of the Pasteur pipettes was sealed by a flame. The air tightened Pasteur pipettes were further heated in a 100 °C water bath for 2 hr. One end of the sealed Pasteur pipettes were cut open to withdraw the hydrolysate. The hydrolysate (2 µl) from each sample was spotted on a TLC plate (Kiesegel 60 F_{254}, Merck). The spots were air dried, and the TLC plate was developed in a fume hood with developing solvent ; pyridine: ethylacetate: dH_2O (26: 66: 8). The sugars on the TLC plate were detected by an alkaline silver staining method (Trevelyan *et al*, 1950). The plate was first sprayed with reagent A (0.1 ml of saturated aqueous silver nitrate was added into 20 ml acetone, and dH_2O was then added drop-wise until the silver nitrate redissolve), and then with reagent B (0.5 M NaOH in aqueous ethanol). The sprayed plate was air dried and the Rf value of any spot detected was measured.

2.2.3.5 Periodate Uptake of F.V. Fractions

The method of Whistler (1965), with slight modifications, was adopted. Iota-Carrageenan, dextran (MW 72,200) and glucose were used as standard. FH, SFAl or standard (0.6 ml) was mixed with 0.6 ml potassium periodate solution (0.045 M). The reaction mixture was kept at room temperature and in dark. At time intervals, 20 µl of the reaction mixture was taken out and diluted 250 times for absorbance measurement. The wavelength used for absorbance measurement was 222.5 nm.

2.2.3.6 Limulus Amebocyte Lysate (LAL) Coagulation Assay

All glass wares used in the assays were heated at 180 °C for 3 hr. Pipette tips and dH_2O were autoclaved. The reconstituted Pyrotell, 0.1 ml, was added to each reaction test tube. To the test tube, test sample (1 mg/ml) was further added, and mixed vigorously for 20-30 seconds. The shaken tubes were placed in a 37 °C ± 1 °C air bath for 60 min. ± 2

min. without disturbance. At the end of the incubation period, the tube was inverted with one smooth motion. A positive test is indicated by the formation of a gel which did not collapse when the tube was inverted.

2.2.3.7 The Digestion of F.V. Fractions with Laminarinase

The method of Czop and Austen (1985),with slight modifications, was adopted. Laminarinase, derived from the algae *Laminaria* sp., was used to check the linkage type of the fractions (FH, SFAI and IFAI). Any glucose released from the digestion was determined by a glucose oxidase kit purchased from Sigma Chem., U.S.A.. Laminarinase was dissolved into citrate buffer (0.005 M citrate + 0.15 M NaCl, pH 5.2) in concentration equals 1 unit/ml buffer. Different *Flammulina* fractions and standards were also dissolved in the same buffer (1 mg/ml). Equal volume of enzyme and substrate were mixed and incubated for 24 hr. to ensure complete digestion (if possible). For every 2 volumes of enzyme digestion mixture, 4 volumes of PBS were added. PBS was used to adjust the pH of the reaction mixture to 7.0 for the action of glucose oxidase. Then 0.1 ml of pH adjusted enzyme digested mixture was taken to determine glucose amount released by the Sigma glucose kit. The percentage of digestible β-(1,3)-D-glucan was calculated according to the following equation.

$$\% \text{ of digestion} = \frac{\text{OD of test sample}}{\text{OD of Laminarin}} 100\%$$

2.2.3.8 Secondary and Tertiary Structure Determination of FH and SFAI

The method of Blaschck *et al* (1992), with slight modifications, was adopted. Other than IR, NMR and X-ray analysis, Congo Red dye provide a cheap, simple and rapid method to determine the conformation of β-(1→3)-D-glucan—the triple helical, the single helical or the random coil structure. The conformation of the glucan helix is maintained by hydrogen bonds which can be disrupted by alkaline. In fact, the triple helical structure can be disrupted into single helix by alkaline pH. A further increase in alkalinity disrupts the single helix into a random coil. The wavelength, where maximum absorption occurred, of Congo Red dye when binds to triple helix and random coil structure is similar to that of unbound dye. However, there are differences in the wavelength value when Congo Red dye binds to a single helical structure. In the assay, 0.375 ml Congo Red solution (3.8 μM) was mixed with 0.75 ml sample (3 mg/ml). NaOH,0.375 ml , of various concentrations (0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M, 1.2 M and 2.0 M) were added to the mixture to study the wavelength shift of the Congo Red dye in different NaOH concentrations. The absorption spectrum of the Congo Red/glucan complexes in different NaOH concentration was scanned from 450 nm to 550 nm and λ_{max} was determined from the absorption spectrum.

2.2.3.9 Molecular Weight Estimation of FH and SFAI

Gel filtration chromatography (Andrews, 1965) was used to estimate the molecular weight of the *Flammulina* fractions (FH & SFAI). Sephadex G-200 swelled in 0.15 M NaCl (the running buffer) was packed in a column of 1.6 cm× 31 cm dimension. The flow rate of the packed column was adjusted to 0.5 ml/min. with a peristaltic pump. The eluent of the column was collected by a fraction collector (Gilson TDC 80) with each tube collected 1.1 ml eluent. The separation capacity of the column was characterised by

Blue Dextran (Average mol. wt. > 2000 kD, 10 mg/ml) and $K_2Cr_2O_7$ (mol. wt. 0.294 kD, 100 mM). Except FH, SFAI and standards (3 mg/ml) were all dissolved in 0.15 M NaCl. The high viscosity of FH make it impossible to be analysed by the chromatographic technique without prior to processing. In the processing, FH (3 mg/ml) was first dissolved in 0.15 M NaOH. The alkaline treatment disrupt the triple helical structure of FH to single helical structure and thus the molecular weight of the molecule should roughly be reduced to one-thirdth of its original. Before applying alkaline treated FH to the chromatographic column, every ml sample was neutralised by 50 µl 3 M HCl. The NaCl concentration of the neutralised sample was approximated 0.15 M. The detection of Blue Dextran, $K_2Cr_2O_7$ and the carbohydrate samples' elution peaks were achieved by measuring O.D. 650, O.D. 400 and O.D. 490 (phenol-sulfuric acid method) respectively.

2.2.3.10 Vascular Dilation and Hemorrhage (VDH) Activity of F.V. Fractions

Male ICR albino mice were divided into groups of ten. The mice were injected intraperitoneally with either 0.2 ml 0.15 M NaCl, FH (2.5 mg/ml), FAI (2.5 mg/ml), SFAI (2.5 mg/ml), IFAI (2.5 mg/ml) or dextran (2.5 mg/ml), for four consecutive days (from day 0 to day 3). The number of mice with VDH response was recorded everyday after the first injection until day 35 was reached.

2.2.4 ISOLATION AND PREPARATION OF CELLS

2.2.4.1 Bone Marrow Cell

Female BALB/c mice were used. Mice were sacrificed by cervical dislocation. The femurs of the mice were removed by aseptic techniques. The femurs were put into a petric dish containing cold RPMI with 10 % FCS. The ends of the femurs were cut open

with a pair of scissors. A 2 ml syringe, connected to a 25 G needle, containing complete medium was used to flush out the marrow plug of the femurs. Cells in the plugs were dispersed by vigorous shaking. Red blood cells present in the cell mixture were removed by Ficoll-paque gradient centrifugation. Bone marrow cells obtained from Ficoll-paque gradient centrifugation were washed twice with RPMI and then resuspended in complete medium (10^6 cells/ml).

2.2.4.2 Peritoneal Exudate Cell (PEC)

Mice were killed by cervical dislocation. The entire body of mouse was immersed in 70 % ethanol. The hair of the abdominal skin was cut and removed. Ice-cold PBS, 5 ml, was injected intraperitoneally. Peritoneal fluid was collected by using a 5 ml syringe fitted with a 20- gauge needle. The peritoneal cavity of mouse was lavaged 2 times. The cells were pelleted by spinning at 300×g for 5 min. at 4 °C and washed with cold PBS twice before use.

2.2.4.3 Splenocytes

Mice were sacrificed by cervical dislocation. The spleens of the mice were removed aseptically, and were cut into small pieces with a pair of fine scissors. The spleen fragments were pressed gently through a sterile 60 mesh stainless steel sieve with a plunger of a 5 ml plastic syringe. Large cell clumps and debris were removed by centrifugation (300×g, 10 seconds). The cell pellet was resuspended in plain RPMI medium and washed three times by centrifugation (300×g, 5 min.). The washed cells were resuspended in complete RPMI medium and kept at 4 °C. The viability of the cell suspension was determined by the tryphan blue dye exclusion method.

2.2.4.4 Depleting Mouse T-cells by Anti-Mouse T-cell Antigen Antibody Plus Complement Treatment

Forty μl anti-Thy 1.2 antibody (20 fold diluted, sterilised by filtering) was added to 2 ml lymphocytes cell suspension (2×10^7 cells/ml) at a final antibody dilution of 1 : 1,000. The mixture was then incubated at room temperature for 30 min. The cells were spun down and resuspended in 2 ml guinea pig complement (diluted 1 : 5 with RPMI medium) for 45 min. at 37 °C to lyse the T-cells. The cells were then washed twice with RPMI and resuspended in complete RPMI medium (5×10^6 cells/ml). The viability of the remaining cells was determined by tryphan blue dye exclusion method.

2.2.4.5 Depleting Mouse B-cells by Cedarlane Column Kit

Cedarlane Column Kit was used to prepare mouse B-cell deplicted lymphocytes, T-lymphocytes. The preparation strictly followed the procedure described in the manual of the Cedarlane mouse T-cell recovery column kit. The Cedarlane mouse T-cell Recovery Column, efficientlly, removed B-cells from mouse lymphocyte population. Greater than 95 % of B-cells were removed from lymphocyte preparations under optimal conditions. The eluted T-cells were fully functional.

2.2.5 ASSAYS FOR THE CYTOTOXICITY OF *Flammulina velutipes*

2.2.5.1 Brine Shrimp Assay

The method of Solis *et al* (1992) was used . Eggs of *Artemia salina*, a kind of brine shrimp, were allowed to hatch in 3.3 % sea water at 26-29 °C for 48 hr. 100 μl suspension of nauplii containing 10-25 organisms was added to each well (triplicate) of a

96-well plate. 100 μl of test sample of various concentrations (samples were dissolved in 3.3 % sea water) was added to the wells. The plate was then covered and incubated at 26-29 °C for 24 hr. Any nauplii in each well that cannot survive during the incubation period were counted under a binocular microscope (× 15). To obtain the total count, 100 μl of 1.5 M HCl was added to each well. After 5 min. of incubation, all survivors were killed by the acid and the total number of shrimps in each well was counted. The % of living shrimp was calculated according to the following equation.

$$\% \text{ of survivor} = \frac{\text{total shrimp count - dead shrimp count}}{\text{total shrimp count}} 100\%$$

2.2.5.2 *In vitro* Cytotoxicity of FH and SFAI on Bone Marrow Cells of Female BALB/c Mice

Bone marrow cells obtained from female BALB/c mice under aseptic conditions were resuspended in RPMI supplemented with 10 % FCS (10^6 cells/ml). Serial dilutions of FH, SFAI and cycloheximide were prepared (50 μg/ml, 100 μg/ml and 200 μg/ml). Test samples or cycloheximde (100 μl) was added to the wells of a 96-well plate. To the well with sample or cycloheximide, 100 μl bone marrow cell suspension was added. The plate was then incubated at 37 °C for 72 hr. in an incubator with 5 % CO_2 supply. After incubation, 0.5 μCi tritiated thymidine/ 50 μl medium was added to each well and the plate was allowed to incubate for another 12 hr. Before harvesting the pulsed cells, the cells were subjected to one cycle of freezing and thawing. The radioactivity of the harvested cells was determined by a liquid scintillation counter.

2.2.5.3 *In vivo* Cytotoxicity of FH and SFAl on Female BALB/c Mice

Female BALB/c mice were divided into groups of 10. Test samples or NaCl (0.2 ml, 2.5 mg/ml 0.15 M NaCl) were injected intraperitoneally. The number of mice, that survive the treatment, was recorded every day after the injection until day 35 was reached.

2.2.6 ASSAYS FOR THE IMMUNOMODULATORY ACTIVITIES of *Flammulina velutipes*

2.2.6.1 *In vitro* Mitogenic Activities of FH and SFAl on Murine Lymphocytes

Lymphocytes obtained from female BALB/c mice were used in the assay. Lymphocytes were resuspended in complete medium (5×10^6 cells/ml). The cell suspension, 100 µl, was added to each well of a 96-well plate. Then 100 µl FH and SFAl of various concentrations (50 µg/ml, 100 µg/ml and 200 µg/ml complete medium) was added to the cells. LPS (final concentration ; 10 µg/ml) and ConA (final concentration ; 5 µg/ml) were used as positive control. The plate was allowed to incubate at 37 °C, with 5 % CO_2 supply, for 48 hr. After the incubation, 5 µCi tritiated thymidine/ 50 µl complete medium was added to the well and allowed to incubate for further 6 hr. The pulsed cells were harvested on a glass fiber filter with a cell harvester, and the radioactivity of the harvested cells was determined with a liquid scintillation counter.

2.2.6.2 *In vitro* Mitogenic Activities of FH and SFAI with PMB on Murine Lymphocytes

Lymphocytes obtained from female BALB/c mice were resuspended in complete medium (1×10^7 cells/ml). Fifty µl of the cell suspension was added to each well of a 96-well plate. Then 100 µl of complete medium, LPS (10 µg/ml) or test samples of various concentrations (50 µg/ml, 100 µg/ml and 200 µg/ml complete medium) was added to the cells. For wells with Polymyxin B (PMB) added , PMB suppressed all or most of the mitogenic activity of LPS. The plate was incubated at 37 °C, with 5 % CO_2 supply, for 48 hr. After the incubation, 5 µCi tritiated thymidine/ 50 µl complete medium was added to the well and allowed to incubate for further 6 hr. The pulsed cells were harvested on a glass fiber filter with a cell harvester, and the radioactivity of the harvested cells was determined with a liquid scintillation counter.

2.2.6.3 *In vitro* Mitogenic Activities of FH on T-cell depleted Murine Lymphocytes

Lymphocytes obtained from female BALB/c mice were resuspended in plain RPMI medium (2×10^7 cells/ml). Forty µl of sterile anti-Thy 1.2 antibody was added to 2 ml cell suspension (final antibody dilution ; 1 : 1,000). The mixture was incubated at room temperature for 30 min. After incubation, the cells were spun down and resuspended in 2 ml sterile guinea pig complement (diluted 1: 5 with RPMI medium) for 45 min. at 37 °C to lyse any T-lymphocytes. After cell lysis, the cells were washed twice with RPMI medium. The B-lymphocytes that survived the lytic procedure were resuspended in complete medium (5×10^6 cells/ml). In this experiment, 100 µl of the B-lymphocyte enriched suspension was added to each well of a 96-well plate. Then 100 µl of the complete medium, FH (200 µg/ml), ConA (2 µg/ml) or LPS (10 µg/ml) was added to the cells. The plate was mixed and was allowed to incubate at 37 °C, with 5 %

CO_2 supply, for 48 hr. After the incubation, 5 μCi tritiated thymidine/ 50 μl complete medium was added to the well and allowed to incubate for further 6 hr. The pulsed cells were harvested on a glass fiber filter with a cell harvester, and the radioactivity of the harvested cells was determined with a liquid scintillation counter.

2.2.6.4 *In vitro* Mitogenic Activities of FH on B-cell depleted Murine Lymphocytes

B-cell depleted lymphocytes were prepared by using the Cedarlane Column Kit (Cedarlane Lab. Ltd., Canada). The T-lymphocytes obtained from the column were washed twice with RPMI, and resuspended in complete medium (5×10^6 cells/ml). In this case, 100 μl of the T-cell enriched suspension was added to each well of a 96-well plate. Then 100 μl of the complete medium, FH (200 μg/ml), ConA (2 μg/ml) or LPS (10 μg/ml) was added to the cells. The plate was mixed and was allowed to incubate at 37 °C, with 5 % CO_2 supply, for 48 hr. After the incubation, 5 μCi tritiated thymidine/ 50 μl complete medium was added to the well and allowed to incubate for further 6 hr. The pulsed cells were harvested on a glass fiber filter with a cell harvester, and the radioactivity of the harvested cells was determined with a liquid scintillation counter.

2.2.6.5 *In vitro* Co-mitogenic Activity of FH and SFAl on Murine Lymphocytes

Lymphocytes isolated from female BALB/c mice were resuspended in complete medium (1×10^7 cells/ml). Fifty μl of the cell suspension was added to each well of a 96-well plate. Then 100 μl of complete medium, or test samples was added to the cells. Furthermore, 50 μl of either complete medium, LPS (20 μg/ml) or ConA (4 μg/ml) was added to the 150 μl cell mixture. The plate was shaken and was allowed to incubate at 37 °C, with 5 % CO_2 supply, for 48 hr. After the incubation, 5 μCi tritiated thymidine/

50 μl complete medium was added to the well and allowed to incubate for further 6 hr. The pulsed cells were harvested on a glass fiber filter with a cell harvester, and the radioactivity of the harvested cells was determined with a liquid scintillation counter.

2.2.6.6 *In vitro* Mitogenic Activities of FH and SFAl on Murine Bone Marrow cells

Bone marrow cells isolated from female BALB/c mice, with aseptic technique, were resuspended in complete medium (10^6 cells/ml). The cell suspension, 100 μl, was added to each well of a 96-well plate. Then 100 μl of complete medium, murine rIL-3 (400 μg/ml) or test samples of various concentrations (50 μg/ml, 100 μg/ml and 200 μg/ml) was added to the wells. The plate was shaken and was allowed to incubate at 37 °C, with 5 % CO_2 supply, for 48 hr. After the incubation, 5 μCi tritiated thymidine/ 50 μl complete medium was added to the well and allowed to incubate for further 12 hr. The pulsed cells were subjected to one cycle of freezing and thawing, before being harvested on a glass fiber filter with a cell harvester. The radioactivity of the harvested cells was determined with a liquid scintillation counter.

2.2.6.7 *In vivo* Mitogenic Activities of FH and SFAl on Murine Lymphocytes

Forty-five female C57BL/6L mice were divided into groups of 15. NaCl (0.15 M), FH (2.5 mg/ml 0.15 M NaCl) or SFAl (2.5 mg/ml 0.15 M NaCl), 0.2 ml, was injected into the peritoneal cavity of the mice on day zero. Five mice from each group were killed on day zero, three and five respectively. Lymphocytes were obtained from the killed mice. The isolated lymphocytes were resuspended in complete RPMI medium (5×10^6 cells/ml).The cell suspension (100 μl) was added into each well of a 96-well

plate. The cells were pulsed with 5 μCi tritiated thymidine (1 μCi/10 μl complete medium) for 6 hr. immediately. The pulsed cells were harvested on a glass fiber with a cell harvester. The radioactivity of the harvested cells was determined with a liquid scintillation counter.

2.2.6.8 Effect of FH and SFAl on the Enhancement of First Antibody Production of SRBC Immunised Mice

Fifteen male ICR albino mice were divided into groups of 5. To each mouse 0.2 ml 0.15 M NaCl, FH (2.5 mg/ml 0.15 M NaCl) or SFAl (2.5 mg/ml 0.15 M NaCl) was injected into the peritoneal cavity on day -2, 0 and 2 respectively. Then 0.2 ml 20 % (vol/vol) sheep red blood cells (SRBC) suspension in PBS was injected into each mice intraperitoneally on day zero. On day 6, serum was obtain from the mice and was checked for its antibody titre by hemagglutination. Hemagglutination was done by adding 50 μl PBS to well 2 to 12 in row A, B, C, D, E and F of a V-shaped 96-well plate (replicate for each sample serum). Fifty μl of test serum was added to well 1 and 2 in row A and B (control serum), C and D (serum from FH treated mice), and E and F (serum from SFAl treated mice). Two-fold serial dilutions of the antiserum was done until a 2048 (2^{11}) fold was reached. Then , 50 μl of 2 % SRBC was added to each well. A negative control was done by adding 50 μl of PBS together with 50 μl of 2 % SRBC to separate wells. The contents of the wells were mixed gently with a plate shaker. After shaking, the plate was allowed to stand at room conditions for 8 hr., and then the hemagglutination end-point recorded. The titre was reported as the highest dilutions of the serum that has no apparent hemagglutination.

2.2.6.9 Effect of FH and SFAI on the *in vitro* Phagocytic Activity of Murine Macrophage

Five female BALB/c mice were killed and their peritoneal cavities were washed with PBS to obtain PEC. The obtained PEC was washed twice with PBS and finally resuspended in Hank's buffer saline with 5 % FCS (10^8 cells/ml). The suspension, 100 µl, was spreaded onto a cover slip, where the cover slip was placed inside a 35× 10 mm culture dish (Falcon, U.S.A.). The cells were incubated at 37 °C, with 5% CO_2 supply, for 4 hr. In the incubation, adherent cell such as peritoneal macrophage, adhered to the glass cover slip. Any non-adherent cells were washed away with Hank's buffer (2.5 ml× 3 times) delivered with a Pasteur pipette. After the removal of non-adherent cells, 2 ml Hank's buffer with 5 % FCS was added into the petri dish. Then 25 µl dH_2O, test sample (1.2 mg/ml) or ConA (2 mg/ml) was added to the petri dish and allowed to incubate for 10 min. Also 100 µl of yeast cell wall suspension (25× 10^7 cells/ml PBS) was added. The mixture was than incubated at 37 °C, with 5% CO_2 supply, for 1 hr. After incubation, non-ingested yeast cell wall was washed away with Hank's buffer. The adherent cells were fixed with methanol for 10 min. After fixation, the cell wall was washed with running water for 15 min. The washed cells were treated with 10 % periodic acid for 10 min. The ingested yeast cell walls in adherent cells were stained by immersing the cells in Schiff's reagent for 30 min. (Dacie and Lewis, 1991). After staining, the cells were rinsed 3 times with rinsing solution (6 ml 10 % sodium metabisulphite + 5 ml 1 M HCl was added to 89 ml dH_2O). The rinsed cells were washed in distilled water for 5 min. The number of adherent cells with yeast cell walls ingested, of 100 adherent cells were counted.

2.2.6.10 Effect of FH and SFAl on the *in vivo* Phagocytic Activity of Murine Macrophage

Twenty-five female BALB/c mice were divided into 5 groups. Each group of mice was injected intraperitoneally either with 0.2 ml 0.15 M NaCl, FH (2.5 mg/ml), FAl (2.5 mg/ml), SFAl (2.5 mg/ml) or IFAl (2.5 mg/ml). Three days after injection, mice were killed and peritoneal cells were washed out with PBS. The washed out PEC were washed twice with RPMI and then resuspended in Hank's buffer saline with 5 % FCS (10^7 cells/ml). The cells were then assayed for the phagocytic activity by the same procedure of that in *in vitro* phagocytosis.

2.2.6.11 *In vivo* Migration of Macrophage in FH- and SFAl-treated Mice

Female BALB/c mice were divided into groups of 5. Mice in each group was injected either intraperitoneally with 0.2 ml 0.15 M NaCl, FH (1.25 mg/ml), SFAl (1.25 mg/ml) or 2 ml 3 % Thioglycollate. Three days after injection, the mice were killed and peritoneal cells were washed out from the mice with PBS. PEC were washed twice with RPMI and then resuspended in RPMI with 10 % FCS (10^6 cells/ml). 2 ml of the cell suspension was added to the wells of 24-well plate. The cells were incubated at 37°C, with 5 % CO_2, for 4 hr. After incubation, non-adherent cells were washed away with PBS. Two ml 0.5 % neutral red (in PBS) was added to the cells, and was allowed to incubate for further 1 hr. at 37°C. Excess neutral red was removed by washing with PBS (2 ml × 2 times). The washed plate was then dried. One % SDS, 600 µl, was added to the dried cells. The added SDS dissolved the cells and neutral red. The absorbance of SDS with neutral red dissolved was measured at 540 nm, and using 1 % SDS as blank.

2.2.6.12 Effect of FH and SFAI on the Enhancement of Murine PEC Cytostatic Activity

Female BALB/c mice were divided into groups of 5. The mice were injected intraperitoneally with either 0.2 ml 0.15N NaCl, FH (2.5 mg/ml) or SFAI (2.5 mg/ml). Three days after injection, the mice were killed and peritoneal cavity was washed with PBS (10 ml/mouse). The wash-out PEC was washed with PBS (15 ml × 2 times). The washed cells were resuspended in PBS (3.2×10^7 cells/ml). Cells suspension of dilutions 2×, 4× and 8× were prepared and 100 µl of the cell suspension was added to 8×10^5 L-929 cells in a 96-well plate. The L-929 cells were added to the wells of the 96-well plate 18 hr. earlier. The cell mixture were then incubated at 37 °C, with 5% CO_2 supply, for 4 hr. After the incubation, the cell mixture was pulsed with 5 µCi tritiated thymidine / 50 µl complete medium for 6 hr. The pulsed cells were harvested on a glass fiber filter with a cell harvester. The radioactivity of the harvested cells was determined with a liquid scintillation counter. The percentage of tumor cell killing was calculated according to the following equation.

$$\% \text{ of killing} = \frac{\text{count of control} - \text{count of test sample}}{\text{count of control}} 100\%$$

2.2.6.13 Effect of FH and SFAI on the Fc Receptor Expression of Peritoneal Exudate Cells

Female BALB/c mice were divided into groups of 5. The mice were injected intraperitoneally either with 0.2 ml 0.15N NaCl, FAI (2.5 mg/ml) or SFAI (2.5 mg/ml). Three days after intraperitoneal injection, mice were killed and peritoneal cells were washed out with PBS. The wash-out peritoneal cells were washed with RPMI (15 ml × 2 times). The washed PEC was resuspended in RPMI containing 10% FCS (2×10^6

cells). Then 0.4 ml PEC suspension was then mixed with 0.4 ml anti-SRBC antibody coated SRBC (3×10^8 cells/ml GVB). The mixture was incubated at 37°C for 15 min. The mixture was mixed for every 5 min. during the incubation. After incubation, cells were spun down (1,000×g , 10 min.) at 4°C. The cell pellet was then resuspended by gentle shaking. The number of 100 PEC, with immune rossetting, was counted.

2.2.6.14 Effect of FH and SFAl on Murine Serum Cytokine Level

Male ICR albino mice were divided into groups of 20. The mice were injected either with 0.2 ml 0.15 M NaCl, FH (2.5 mg/ml) or SFAl (2.5 mg/ml) on day 0, 1, 2 and 3. On day 1, 6, 9 and 12, 5 mice were selected from each group and serum was obtained from the mice. The serum collected was tested for GM-CSF, IL-1 α, IL-4 and IFN-γ level by ELISA kits.

2.2.6.15 Effect of FH and SFAl on Murine Serum TNF Level

Female BALB/c mice were divided into groups of 5. Each group of mice may be injected intravenously with 0.2 ml 0.15N NaCl, 0.2 ml FH (1.25 mg/ml 0.15 M NaCl), 0.2 ml SFAl (1.25 mg/ml 0.15 M NaCl) or 0.2 ml LPS (25 μg/ml 0.15 M NaCl). One hr. after injection, blood was obtained from the injected mice. The blood was allowed to clot in room conditions. Any blood clot formed was spun down at 3,500 rpm for 5 min. The supernatant /serum was removed and was subjected to 56 °C treatment of 30 min. The heat treated serum was filtered with 0.45 um pore size sterile filter unit. The serum was 10 fold diluted with medium containing 5 % HIFCS and cycloheximide (10 μg/ml medium). To assay the level of TNF in the serum, L-929 tumor cell, which is sensitive to TNF, was used. L-929 tumor cell suspension (4×10^5 cells/ml), 100 μl, was added to

each well of a 96-well plate, for 18 hr. at 37°C with 5 % CO_2 gas supply, before diluted serum was added. After the incubation, medium was removed by suction. The serum was 2 fold serial diluted until a dilution of 16384 fold was reached. The diluted serum, 100 µl, was added to the well (duplicated for each concentration), and was allowed to incubate for 24 hr. At the end of the incubation, serum was removed by suction, and the cells were washed twice with PBS. Viable cells were stained with Neutral Red. Fifty µl neutral red (0.3 %, wt/vol) was added to each well and then incubated for 1 hr. at 37°C. Excess neutral red was removed by washing the cells twice with PBS. The washed cells were allowed to stay at room conditions. One % SDS, 100 µl, was added per well and was mixed. The 1% SDS dissolved the cells and neutral red. The absorbance of each well was then measured by an ELISA reader (Bio-Rad, USA) at wavelength 540 nm. The percentage of killing was calculated as follow :

$$\% \text{ of killing} = \frac{\text{OD of control - OD of test sample}}{\text{OD of control}} 100\%$$

2.2.6.16 Effect of FH and SFAl on the Augmentation of SRBC lysing ability of Murine Serum

Fifteen male ICR albino mice were divided into groups of 5, and 0.2 ml 0.15 M NaCl, FH (2.5 mg/ml 0.15 M NaCl) or SFAl (2.5 mg/ml 0.15 M NaCl) was injected into peritoneal cavity of each mouse on day -2, 0 and 2 respectively. Then 0.2 ml 20 % (vol/vol) sheep red blood cells (SRBC) suspension in PBS was injected into each mouse intraperitoneally on day zero. On day 6, serum was obtain from the mice and was checked for its complement level. The serum complement level was monitored by checking the degree of SRBC lysis by the serum. The serum, 0.2 ml, was mixed with 0.2 ml 2 % SRBC in PBS. The mixture was shaken gently, and was allowed to stand at room

temperature for at least 8 hr. Any surviving intact SRBC was spun down (300×g, 5 min.), and 0.3 ml of the supernatant was removed and mixed with 0.3 ml PBS. The absorbance of the supernatant was measured at wavelength 541 nm.

2.2.7 ASSAYS FOR THE ANTI-TUMOR ACTIVITIES OF *Flammulina velutipes*

2.2.7.1 *In vitro* Anti-tumor Activity of FH and SFAI

Tumor cells, PU5-1.8 and Sc-180, grew into logarithmic phase were used in the assay. The cells were washed twice with plain RPMI under sterile conditions. Washed cells were resuspended in complete medium (10^6 cells/ml). The cell suspension, 100 μl, was added into each well of a 96-well plate. Fractions to be tested were diluted to various concentration (50 μg/ml, 100 μg/ml and 200 μg/ml) with complete medium, and filtered with a 0.4 μm pore size sterile filter unit. The carbohydrate fractions, 100 μl, of various concentrations was added to the well that contain tumor cells (triplicate). The plate was then allowed to be incubated at 37°C for 48 hr. in a CO_2 supplied incubator. After the incubation, 5 μCi tritiated thymidine/50 μl complete medium was added to each well and then further incubated for 6 hr. The pulsed cells were harvested with a cell harvester and the radioactivity of the harvested cell was determined by a liquid scintillation counter. The percentage of suppression of the tumor growth was calculated according to the following equation:

$$\% \text{ of killing} = \frac{\text{count of control} - \text{count of test sample}}{\text{count of control}} 100\%$$

In order to confirm the results of the *in vitro* antitumor activity (monitored by thymidine incorporation) of FH and SFAI, MTT assay (Hussain, 1993) was performed as well. The assay was done by adding 50 µl of the cell suspension to each well of a 96-well plate. Carbohydrate fractions to be tested were diluted to various concentrations (50 µg/ml, 100 µg/ml and 200 µg/ml) with complete medium, and filtered with a 0.4 µm pore sterile filter unit. Then 50 µl of the sample carbohydrates of various concentration was added to the well that contain the cells (triplicate). The plate was incubated at 37 °C for 48 hr. in a CO_2 supplied incubator. At the end of the incubation, 30 µl MTT solution (5 mg/ml PBS) was added to each well and incubated for further 1 hr. at 37°C in CO_2 supplied incubator. After this incubation, 100 µl acidified isopropanol (with 0.04 M HCl) was added. Mixing of the well mixture was done by repeated pipetting. The absorbance of the well mixture was read at wavelength 540 nm with a ELISA reader (Bio-Rad, USA). The percentage of suppression was calculated according to the following equation.

$$\% \text{ of killing} = \frac{\text{OD of control - OD of test sample}}{\text{OD of control}} 100\%$$

2.2.7.2 Effect of FH and SFAI on the Growth of Murine Transplantable Tumor *in vivo*

PU5-1.8 tumor cells (a syngeneic tumor) and Sc-180 tumor cells (an allogeneic tumor) were used in the assay. PU5-1.8 tumor cells were grown in female BALB/c mice and Sc-180 tumor cells in male ICR albino mice. The tumors were maintained in the mice as ascitic cells. In case of the syngeneic tumor, 7-day old PU5-1.8 tumor cells (0.5 ml 2×10^6 cells/ml PBS) were injected into the peritoneal cavity of female BALB/c mice on day 0. The mice were also injected with 0.2 ml 0.15 M NaCl, FH (2.5 mg/ml), FAI

(2.5 mg/ml) or SFAI (2.5 mg/ml) on day -2, -1, 0, 1 and 2. On day 7, the regression of the PU5-1.8 tumor was monitored by counting the viable cells of the peritoneal wash. For the allogeneic tumor, 7-day old Sc-180 tumor cells (0.05 ml 2×10^7 cells/ml) were injected into male ICR albino mice subcutaneously at the right groin on day 0. The mice were also injected with 0.2 ml 0.15 M NaCl, FH (2.5 mg/ml), FAI (2.5 mg/ml), SFAI (2.5 mg/ml) or IFAI (2.5 mg/ml) on day 1, 3, 5, 7 and 9. On day 20, mice with no noticeable size of tumor were removed. Thirty days after the tumor injection, the solid tumors were excised and weighted.

2.2.8 STATISTICAL ANALYSIS

All results were expressed as the arithmetic mean ± standard error (S.E.). Student's 't' test was used to determine the confidence limits in population comparisons. $P< 0.05$ was regarded as significant different.

Screening, Purification, Fractionation and Characterisation of β-(1→3)-D-Glucan(s) from *Flammulina velutipes*

CHAPTER THREE

SCREENING, PURIFICATION, FRACTIONATION AND CHARACTERISATION OF β-(1→3)-D-GLUCAN(S) FROM *Flammulina velutipes*

Introduction

In modern world, the sophisticated applications of fungi has bloomed into many different areas. They are the basis of a number of food industrial processes involving fermentation such as the making of bread, wines, beers, the fermentation of cacao bean, the preparation of certain cheeses and many organic acids. In academic area, fungi are powerful tools in biochemical, cytological and genetical studies. In medical area, fungi are important tools in the production of many complex drugs such as ergometrine and cortisone (Jong and Donovick, 1989) and some vitamin preparations, and are responsible for the manufacture of a number of antibiotics, notably penicillin and griseofulvm (Jong and Donovick, 1989). Other than these, in the last 4 decades, the applications of fungi in the treatment of cancer was actively elucidated by many researchers. The earliest use of fungi in cancer treatment may be represented by the work of Protti (1946) who described the lytic action of several species of *Saccharomyces* on transplanted carcinoma by a process which he called " cytophotolysis ". In later years, many different antitumor substances were isolated from different species of fungi. The substances are very diverse in their nature (Jong and Donovick, 1989). The antitumor polysaccharides obtained from fungi represent a unique group of antitumor substances as the polysaccharides are quite homogeneous with respect to their structures. For example, most of the polysaccharides were homopolymer of glucose with the glucose units linked together by β-(1→3)-D-linkage. Furthermore, they show similar antitumor mechanisms.

Results

3.1 SCREENING OF β-(1→3)-D-GLUCAN

Three types of mushroom (fruiting bodies); *Flammulina velutipes*, *Lyophyllum aggregatum* (*shimeji*) and *Volvariella volvacea* , as shown in Fig. 3.1 were selected for the screening of the presence of β-(1→3)-D-glucans. The lyophylised powder of the raw fruiting bodies and the cell wall materials of the mushrooms were screened with aniline blue staining method. As shown in Table 3.1, only the powder of *Flammulina velutipes* and *Volvariella volvacea* were positive to the present of β-(1→3)-D-glucans. The degree of staining of the powder of *Flammulina velutipes* and *Volvariella volvacea* was similar. When the cell wall of *Flammulina velutipes* and *Volvariella volvacea* were stained, only *Flammulina velutipes* was positive. When the degree of staining of the powder and the wall of *Flammulina velutipes* was compared, the wall materials was stained much stronger.

3.2 EXTRACTION AND FRACTIONATION OF *Flammulina velutipes*

The lyophylised mushroom powder make up of around 9-10 % of the fresh weight (fruiting bodies). After extensive wash of the powder, the cell wall materials was obtained. When lyophylised, the weight of the wall materials contribute to around 20 % of the weight of the powder. Hot water extraction of the wall materials release water soluble glucan(s). The released glucan(s) when dialysed and lyophylised yielded the FH fraction. The weight of the FH fraction composed of around 0.5 % that of powder. The hot water extracted cell wall when soaked into alkaline urea solvent released the FAI fraction. The neutralised, dialysed and lyophylised FAI (contains SFAI and IFAI) weighed around 3 % that of the powder. SFAI was separated from IFAI in FAI by centrifugation. The

Fig. 3.1 A diagram showing the fruiting body of the mushrooms; *Flammulina velutipes*, *Lyophyllum aggregatum* (*shimeji*) and *Volvariella volvacea*.

Table 3.1 The screening of the presence of β-(1→3)-D-glucan in three edible mushrooms: *Flammulina velutipes*, *Lyophyllum aggregatum* (*shimeji*) and *Volvariella volvacea*.

Sample		Degree of staining
Flammulina velutipes	Crude powder	++
	Cell wall materials	++++++
Lyophyllum aggregatum	Crude powder	-
	Cell wall materials	-
Volvariella volvacea	Crude powder	++
	Cell wall materials	-
Yeast	Crude powder	++
	Cell wall materials	++++
α-cellulose	/	/
	Cell wall materials	-

The solid samples were stained with aniline blue dye (1 mg/ ml). The stained samples were than destained twice with methanol and dH_2O.

- ve represents no observable staining. + ve represents observable staining. The greater the number of + ve sign the more intense the staining observed. / means the sample had not been determined.

lyophylised SFAl and IFAl weighted about 0.6 and 2.6 % of that of the powder respectively. The results are shown in Table 3.2.

3.3 THE DETERMINATION OF CARBOHYDRATE CONTENT OF F.V. FRACTIONS

The carbohydrate content of the extracted fractions were estimated with the phenol- sulfuric acid method. Dextran, 1 mg/ml, in dH_2O was used as the standard. FH , FAl, SFAl and IFAl were estimated to contain carbohydrate 99.8 ± 10.2 %, 102.8 ± 10.6 %, 102.7 ± 6.1 % and 105.1 ± 0.1 % respectively. The results were listed in Table 3.3.

3.4 THE DETERMINATION OF PROTEIN CONTENT OF F.V. FRACTIONS

Bradford Protein microassay (Bradford, 1976) was adopted instead of Folin-Lowry method (Lowry *et al*, 1951) . In fact, Folin-Lowry reagent caused the polysaccharide(s) of SFAl to precipitate. Futhermore, the protien value determined by the Folin-Lowry method is affected by the present of carbohydrate but not the Bradford method. In Bradford protein microassay, BSA was used as standard. Since IFAl is insoluble, its protein content can't be determinated by this method. When FH and SFAl were tested the protein content of both fractions was found to be about 3 %. These results agreed with that of carbohydrate determination (e.g. nearly 100 % carbohydrate content was estimated). The results were shown in Table 3.4.

Table 3.2 The yield of different polysaccharide fractions isolated from the mushroom *Flammulina velutipes*.

Sample	Mass (g)	Percentage yield (%)		
Fresh mushroom	600.00	100.00		
Lyophylised powder	55.00	9.17	100.00	
Cell wall materials	10.30		19.87	
Lyophylised FH	0.28		0.51	
Lyophylised FAI	1.80		3.27	100.00
Lyophylised IFAI	0.35		0.64	19.57
Lyophylised SFAI	1.41		2.56	78.29

Dry powder of *Flammulina velutipes* were washed twice with PBS (1 g/ ml), ddH_2O, methanol-chloroform, acetone, and ddH_2O (3 g/ ml) to yield cell wall materials. FH was released from the wall materials by hot water extraction (1 g/ ml, 121 ^oC, 30 min.). Solid residues, from the hot water extraction, was dried. FAI was released from the dry residues by alkaline-urea mixture (1 g/ ml, 65 ^oC, 90 min.). The alkaline solution containing FAI was neutralised by acetic acid and then dialysed to remove salt. The dialysed FAI contained SFAI and IFAI. IFAI was separated from SFAI by centrifugation (3,000 × g, 10 min.).

Table 3.3 The carbohydrate content of different polysaccharide fractions isolated from *Flammulina velutipes*; FH, FAl, SFAl and IFAl.

Sample (1 mg/ml dH_2O)	Percentage of carbohydrate (%)
Dextran (M.W. 72,200)	100.0
FH	99.8 ± 10.2
FAl	102.8 ± 10.6
SFAl	102.7 ± 6.1
IFAl	105.1 ± 0.1

The carbohydrate content was determined by phenol-sulfuric acid method (Dubois, 1956) and absorbance was read at 490 nm using dextran (M.W. 72,200) as a standard.

Table 3.4 The protein content in different polysaccharide fractions isolated from *Flammulina velutipes*.

Sample (μg/ ml)	Protein amount (μg)	Percentage of protein (%)
BSA (20)	20	100.0
FH (1000)	30.3 ± 1.0	3.0 ± 0.1
SFAl (1000)	15.1 ± 0.3	1.5 ± 0.0
IFAl (1000)	/	/

The protein content was determined by Bradford method (Bradford, 1976) and absorbance was read at 595 nm using BSA as a standard.

/ means the sample cannot be determined by the method as the sample was a water insoluble solid.

3.5 THE DETERMINATION OF URONIC ACID CONTENT OF F.V. FRACTIONS

The method adopted was widely used but with disadvantages. Under the experimental conditions, neutral sugars at similar concentrations show 10 % interference. In the estimation, D-glucuronic acid was used as standard and, D-glucose (0.5 mg/ml) and Dextran (M.W. 72,200, 0.5 mg/ml) were used as control. The absorbance of , D-glucose and Dextran, which were expected to be free of uronic acid, were shown in Table 3.5. The absorbance of SFAl was similar to that of Dextran. However, both FH and IFAl had a lower value.

3.6 THE DETERMINATION OF SUGAR COMPONENT OF F.V. FRACTIONS

The sugar components of the fractions were determined with a Thin Layer Chromatographic method. The solvent system adopted was efficient in separating various monosugars such as glucose, mannose, galactose, arabinose, fucose and fructose, as shown in Fig. 3.2. The solutions from acid hydrolysis of glucose, SFAL, IFAl, FH and β-(1→3)-D-glucan (from *Pluerotus ostreatus*) when tested with thin layer chromatographic method all the samples showed a single spot. Furthermore, the spot of the sample tested has nearly the same Rf value as shown in Fig. 3.3.

3.7 PERIODATE UPTAKE OF F.V. FRACTIONS

From previous experimental determination, the fractions extracted contain mostly glucose as the constituents. In order to determine the linkage of the glucose units in the polysaccharide(s). Periodate oxidation was performed as a preliminary study. Glucose,

Table 3.5 The uronic acid content of different polysaccharide fractions isolated from the mushroom, *Flammulina velutipes*.

Sample (1 mg/ ml)	Absorbance (525 nm)	Percentage of absorbance with respect to Dextran (%)	Uronic acid equivalent (μg)	Expected uronic acid amount (μg)
Dextran	0.603 ± 0.004	100.0	53.81 ± 0.40	0
D-glucose	0.608 ± 0.005	100.8 ± 0.8	53.40 ± 0.27	0
FH	0.514 ± 0.039	85.2 ± 6.5	45.53 ± 3.46	/
SFAI	0.607 ± 0.024	100.7 ± 3.9	53.75 ± 2.08	/
IFAI	0.549 ± 0.067	91.0 ± 11.1	48.63 ± 5.86	/

The uronic acid content was determined by the carbazole reaction (Chaplin, 1986) and absorbance was measured at 525 nm.

/ means that the samples do not have any expected uronic acid value.

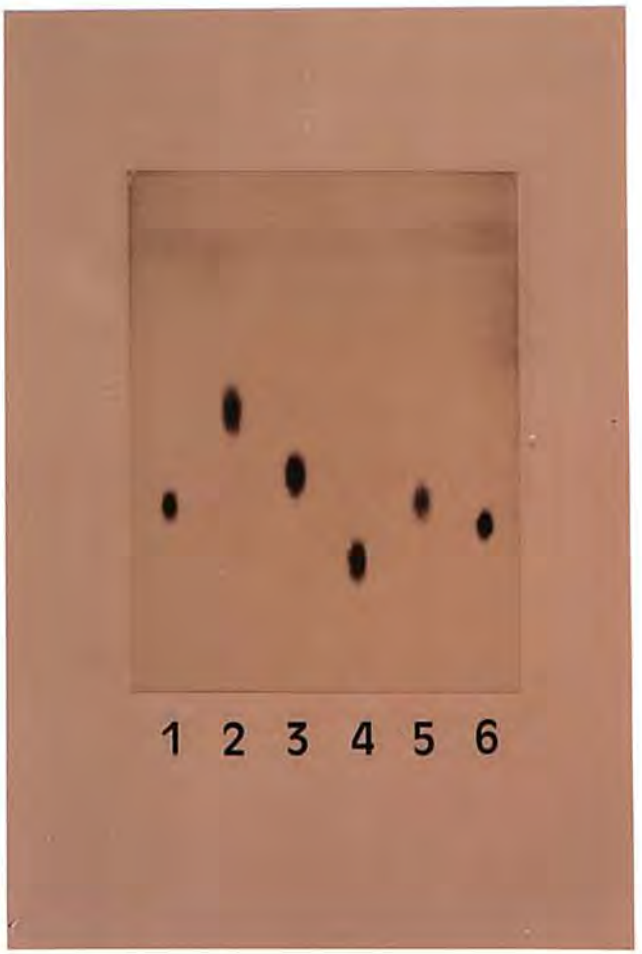


Fig. 3.2 The separation of various monosugars by Thin Layer Chromatography , Lane 1, fructose; Lane 2, fucose; Lane 3, Arabinose; Lane 4, galactose; Lane 5, mannose; and Lane 6, glucose. The monosugars (3 mg/ ml) were spotted onto the TLC plate and separated by developing solvent (pyridine: ethylacetate: dH_2O ; 26: 66: 8). The locations of the sugars were revealed by alkaline sliver staining method.

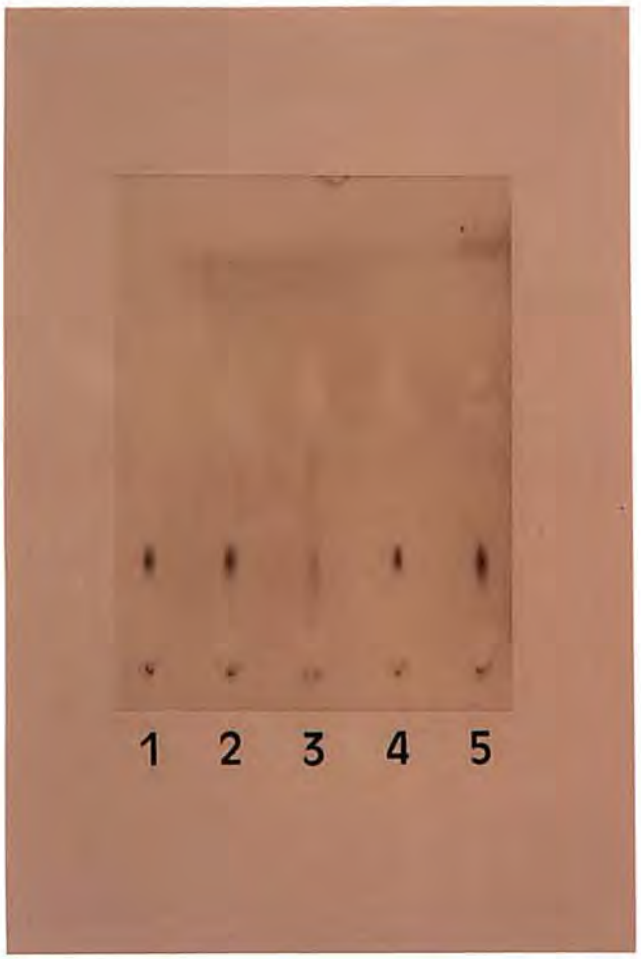


Fig. 3.3 The separation of acid hydrolysate of β-(1→3)-D-glucan (*Pleurotus ostreatus*), FH, IFAl, SFAl and glucose by Thin Layer Chromatography , Lane 1, β-(1→3)-D-glucan (*Pleurotus ostreatus*); Lane 2, FH; Lane 3, IFAl; Lane 4, SFAl; and Lane 5, acid treated glucose. The samples (3 mg/ ml) were hydrolysed with 4 N HCl at 100 ^{o}C for 2 hr.. The hydrolysates were spotted onto the TLC plate and separated by developing solvent (pyridine: ethylacetate: dH_2O , 26: 66: 8). The locations of the sugars were revealed by alkaline silver staining method.

Dextran (M.W. 72,200) and Iota-carageenan were used as a standard control. Fig. 3.4, 3.5 and 3.6 showed the structure of glucose, dextran, polysaccharide of exclusively β-(1→3)-D-linkage and iota-carrageenan. As shown in Fig. 3.7, Glucose standard as expected had very high periodate consumption. For example, glucose consumed about 85 % of periodate in the experimental conditions (assumed 100 % periodate consumption). Dextran had about 67 % consumption and Iota-carageenan had no more than 6 % consumption with respect to glucose. For FH and SFAl, both have about 26 % periodate consumption with respect to glucose.

3.8 LIMULUS AMEBOCYTE LYSATE (LAL) COAGULATION ASSAY

Limulus Amebocyte lysate when encounter LPS or β-(1→3)-D-glucan coagulate to form gel. The fractions were found to have no significant amount of LPS. When the fractions were incubated with LAL all the fractions caused coagulation. The results were shown in Table 3.6.

3.9 THE DIGESTION OF F.V. FRACTIONS WITH LAMINARINASE

The enzyme used in the digestion was Laminarinase which is derived from a brown algae, *Laminaria* sp.. The degree of digestion was expressed as the percentage of digestion with respect to the digestion of Laminarin—a linear β-(1→3)-D-glucan. The degree of digestion of Laminarin was assigned 100 %. The degree of digestion of β-(1→3)-D-glucan (from Barley) was close to that of Laminarin. The digestion of FH, SFAl and IFAl gave 49.8 %, 88.4 % and 13.9 % respectively. The results are shown in Fig. 3.8.

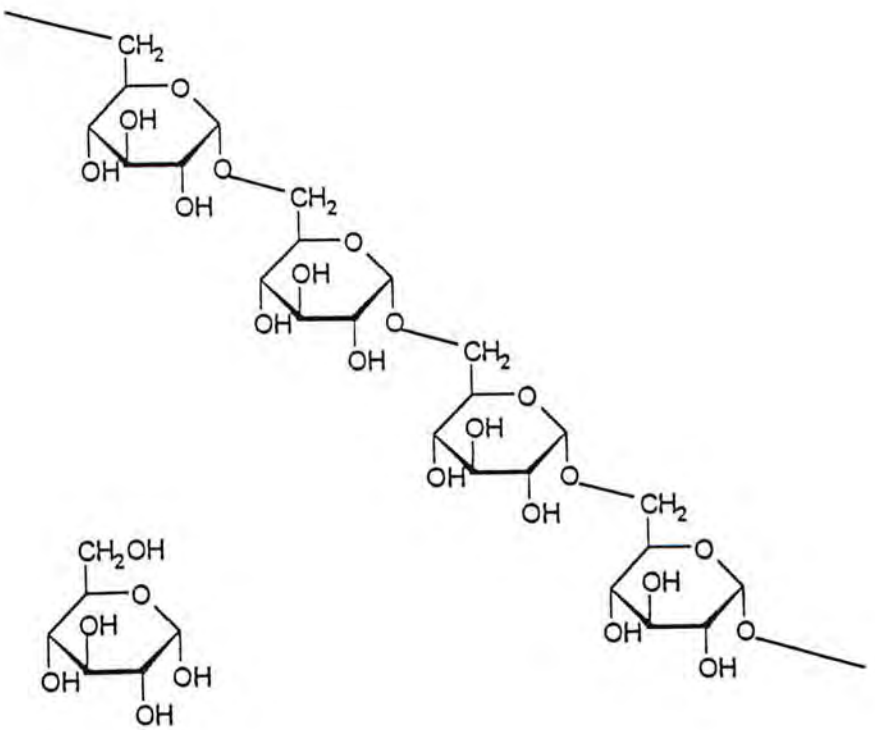
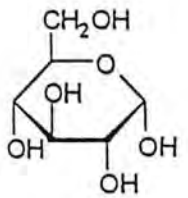
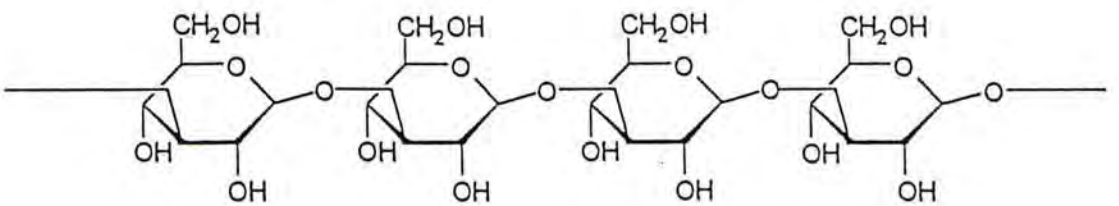


Figure 3.4 Chemical structure of D-glucose and Dextran.

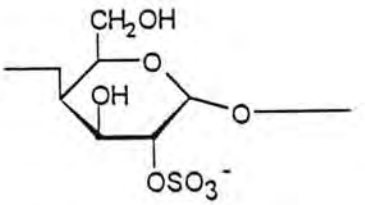


D-glucose

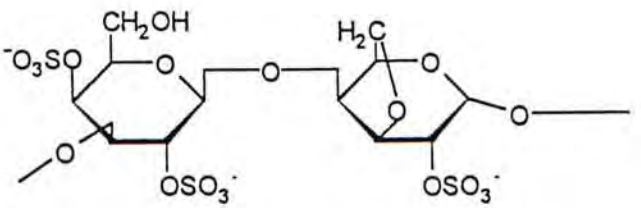


Polysaccharide of beta-(1,3)-D-linkage

Fig. 3.5 Chemical structure of D-glucose and polysaccharide of β-(1→3)-D-linkage.



D-galactose 2-sulfate unit



D-galactose-4-sulfate and 3,6-anhydro-D-galactose 2-sulfate dimeric repeating units

Fig. 3.6 Chemical structure of Iota-carrageenan which is made up of D-galactose-4-sulfate and 3,6-anhydro-D-galactose 2-sulfate with the latter residues replaced by about 10% D-galactose 2-sulfate residues.

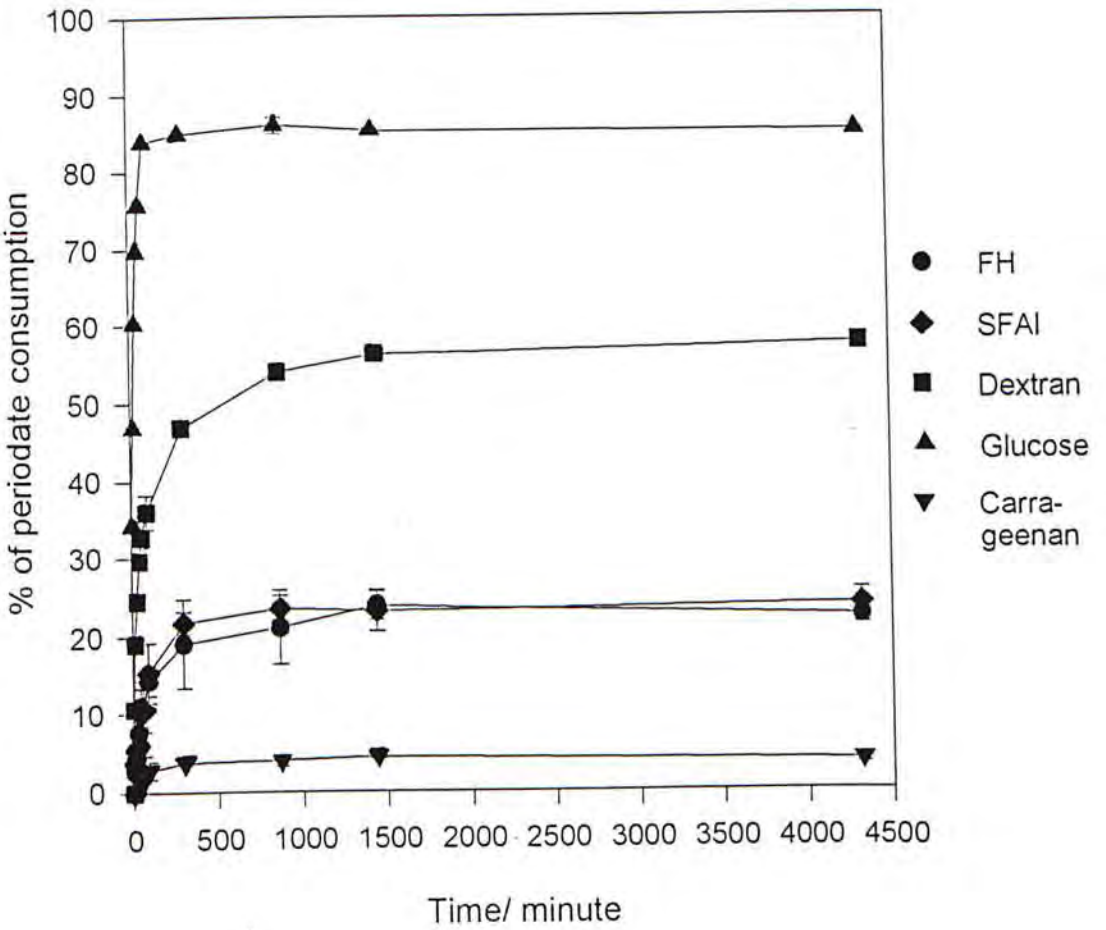


Fig. 3.7 The oxidation of FH and SFAI by potassium periodate. Test sample (3 mg/ ml) of 0.6 ml was mixed with 0.6 ml potassium periodate solution (0.045 M). The reaction mixture was kept under dark at room temperature. At time intervals, 20 µl of the reaction mixture was taken out and diluted 250 times for absorbance measurement. The absorbance was measured at 222.5 nm. Vertical bar represents one standard error.

Table 3.6 The ability of different isolated polysaccharide fractions, FH, FAl, SFAl and IFAl, to initiate the coagulation of Limulus amoebocyte lysate.

Sample (μg/ ml dH_2O)	Coagulation		
	Trial 1	Trial 2	Trial 3
dH_2O	-	-	-
β-(1→3)-D-glucan (1000)	+	+	+
FH (1000)	+	+	+
FAl (1000)	+	+	+
SFAl (1000)	+	+	+
IFAl (1000)	+	+	+

Pyrotell Limulus Amebocyte Lysate reagent was mixed with test samples. The reaction mixtures were placed at 37 °C ± 1 °C for 60 min. ± 2 min.. A positive test was indicated by the formation of a gel which did not collapse when the reaction tube was inverted.

+ sign represents coagulation occurred and - sign represents no coagulation occurred. The β-(1→3)-D-glucan used as positive control was derived from *Pleurotus ostreatus*.

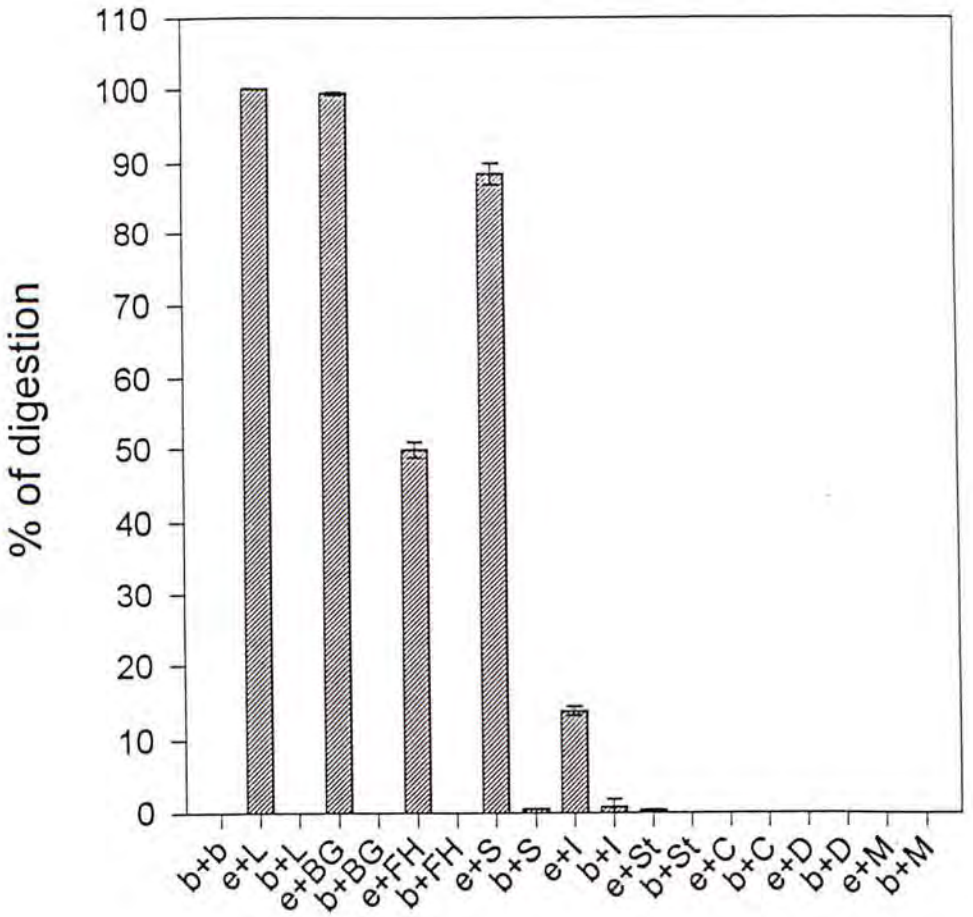


Fig. 3.8 The digestion of F.V. fractions by Laminarinase. Samples (1 mg/ ml) were digested with Laminarinase (vol/vol : 1/1) in citrate buffer (0.005 M citrate + 0.15 M NaCl, pH 5.2) for 24 hr. The pH of the digested mixtures were adjusted by PBS to 7.0 before assaying the amount of glucose released during the digestion. The amount of glucose was assayed by a glucose oxidase kit (Sigma Chem., U.S.A.). Vertical bar represents one standard error.

b — buffer, e — enzyme, BG — barley β-D-glucan, C — α-cellulose, D — dextran, I — IFAl, L — laminarin, M — mannan, S — SFAl and St — starch

3.10 SECONDARY AND TERTIARY STRUCTURE DETERMINATION OF FH AND SFAL

The experiments previously done aimed at the determination of the primary structure of the extracted polysaccharide fractions, FH, SFAl and IFAl. The present method allow the determination of secondary structure. It was found that most β-(1→3)-D-glucan can attain either a random coil, single helical or triple helical structure. The type of structure involved can be determined from the change in value of wavelength where peak absorbance occurred (λ maximum) of Congo red dye. The results are shown in Fig. 3.9. The λ maximum shift of FH/Congo Red dye complex is a characteristic of β-(1→3)-D-glucan with a triple helical structure. When NaOH concentration increase from 0.0 to 0.05 there was not much change in λ maximum (from 498.5 to 497.0) as the glucan retaining a triple helix structure. Further increase in NaOH concentration from 0.05 to 0.15, the λ maximum of Congo red increased markedly (e.g., from 497.0 nm to 502.0 nm). The increased NaOH concentration disrupted the hydrogen bonds which held the triple helical structure, and resulted in the formation of a single helix. The single helix when bind to Congo Red dye caused an increase in λ maximum. When NaOH concentration increased from 0.15 M to higher concentration the λ maximum drop drastically (from 504.0 to 481.5). The high NaOH concentration disrupted the hydrogen bonds that held the single helical structure, and result in a random coil structure. This random coil structure when bound to Congo Red dye result in the decreased λ maximum value. The λ maximum shift curve of SFAl is a characteristic of β-(1→3)-D-glucan with a single helical structure. NaOH concentration when increased from 0.00 to 0.15 M the λ maximum had already in value beyond 500.0 nm (e.g. change from 522.5 to 515.9). When further increased the NaOH concentration the λ maximum dropped drastically (e.g. from 515.9 to 486.7). However, other polysaccharides which do not have helical structures will have λ maximum curve behave similar to that of dH_2O.

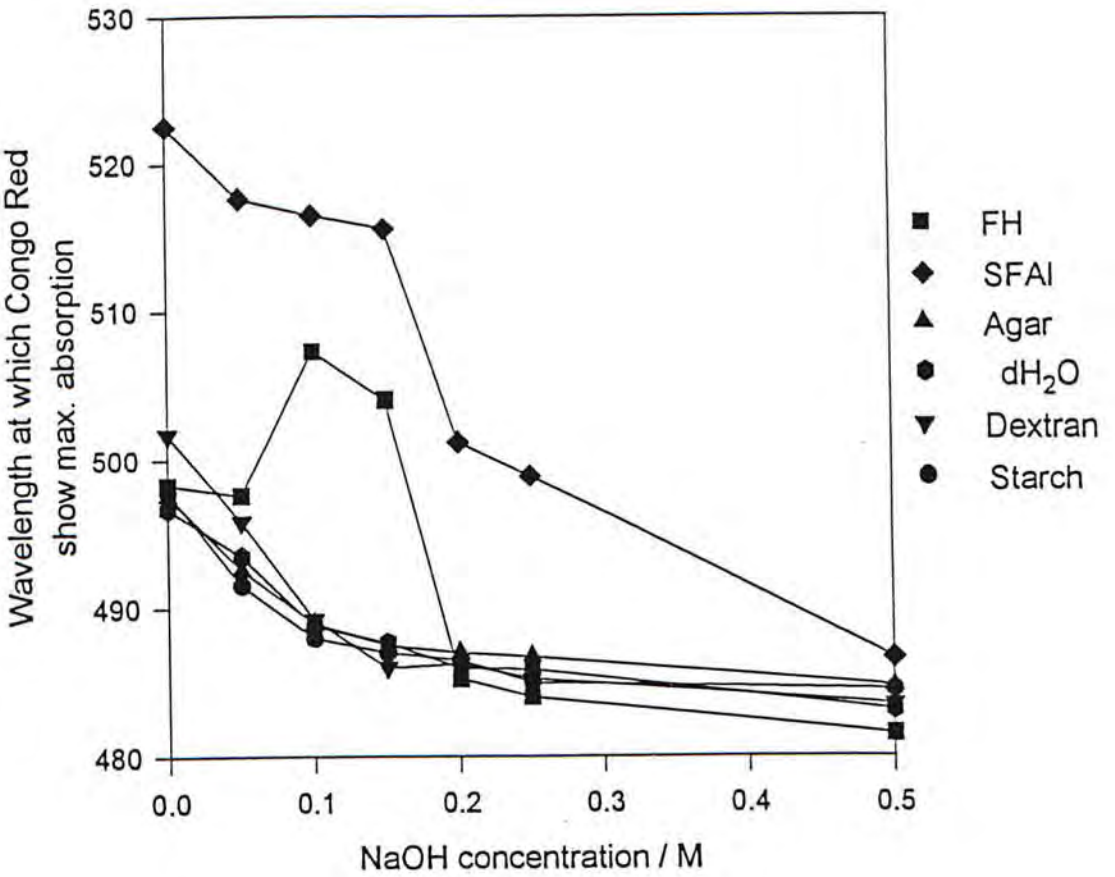


Fig. 3.9 The dependence of the λ_{max} of Congo Red-glucan complexes on the concentration of NaOH. Congo Red solution (3.8 μM) of 0.375 ml was mixed with 0.75 ml sample (3 mg/ ml). NaOH, 0.375 ml, of various concentrations (0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M, 1.2 M and 2.0 M) were added to the mixture to study the λ_{max} shift of the Congo Red-glucan complexes in different NaOH concentrations. The absorption spectrum of the complexes in different NaOH concentration was measured from 450 nm to 550 nm. λ_{max} was determined from the absorption spectrum.

3.11 MOLECULAR WEIGHT ESTIMATION OF FH AND SFAI

The molecular weight of various polysaccharide fractions were estimated with gel permeation chromatography. Sephadex G-200 was used. The results were shown in Fig. 3.10 and 3.11. Fig. 3.10 shows the elution profile of Blue dextran and $K_2Cr_2O_7$, and Fig. 3.11 shows the elution profile of FH and SFAI. The elution volume of Blue dextran represent the void volume of the column and that of $K_2Cr_2O_7$ represent the bed volume of the column. From Fig. 3.10, the void volume should be close to volume 23 ml. Figure 3.11 shows that both FH and SFAI have elution peak at elution volume of 22-23 ml, which was very close to the void volume. In the elution profile of FH, there exist a major peak and a minor peak in between the void and the bed volume. The major peak represents about 66 % and the minor peak represent about 34 % of the FH sample. When FH was eluted with column of identical conditions, except the Sephadex gel used was G-100, there appeared single peak and the peak was eluted at the void volume as shown in Fig. 3.12 and 3.13.

3.12 VASCULAR DILATION AND HEMORRHAGE ACTIVITY (VDH) OF FH, SFAL AND IFAL

This response was reported to have high correlation with antitumor activity of β-(1→3)-D-glucans. β-(1→3)-D-glucans which showed VDH response were expected to be active against Sc-180 solid tumor *in vivo*. Male ICR albino mice were used in the assay. The assay lasted for 35 days and the results are shown in Fig. 3.14 and 3.15. The response mainly localised at the ear of the mice. As clearly shown in Fig. 3.14, the inflammatory response caused extensive blood vessel dilation and hemorrhage. From

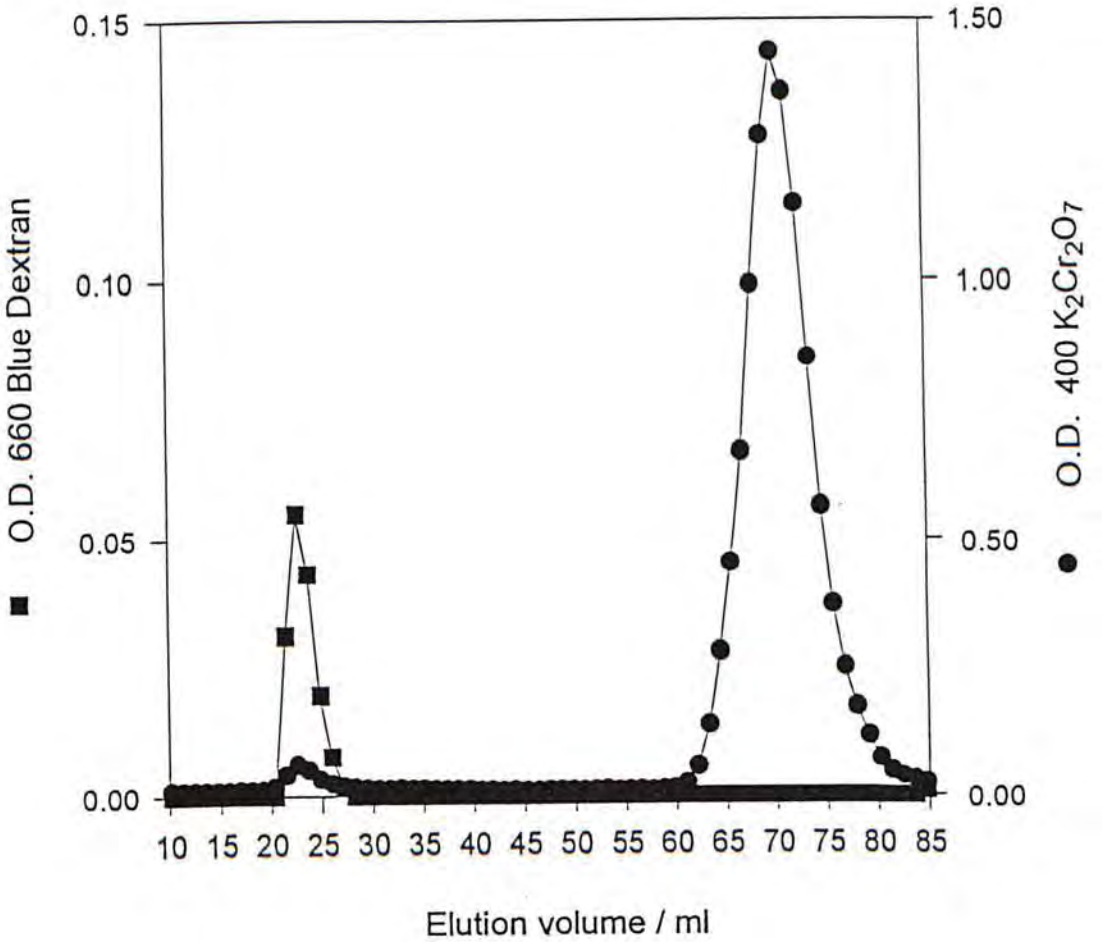


Fig. 3.10 Gel permeation (Sephadex G-200) of Blue Dextran and $K_2Cr_2O_7$. NaCl (0.15 N) was used as the running buffer. The flow rate of the column was adjusted to 0.5 ml/ min. with a peristaltic pump. The eluent of the column was collected by a fraction collector (1.1 ml/ tube). Blue Dextran in the eluent was monitored by measuring asorbance at 660 nm and $K_2Cr_2O_7$ in the eluent was monitored by measuring asorbance at 400 nm.

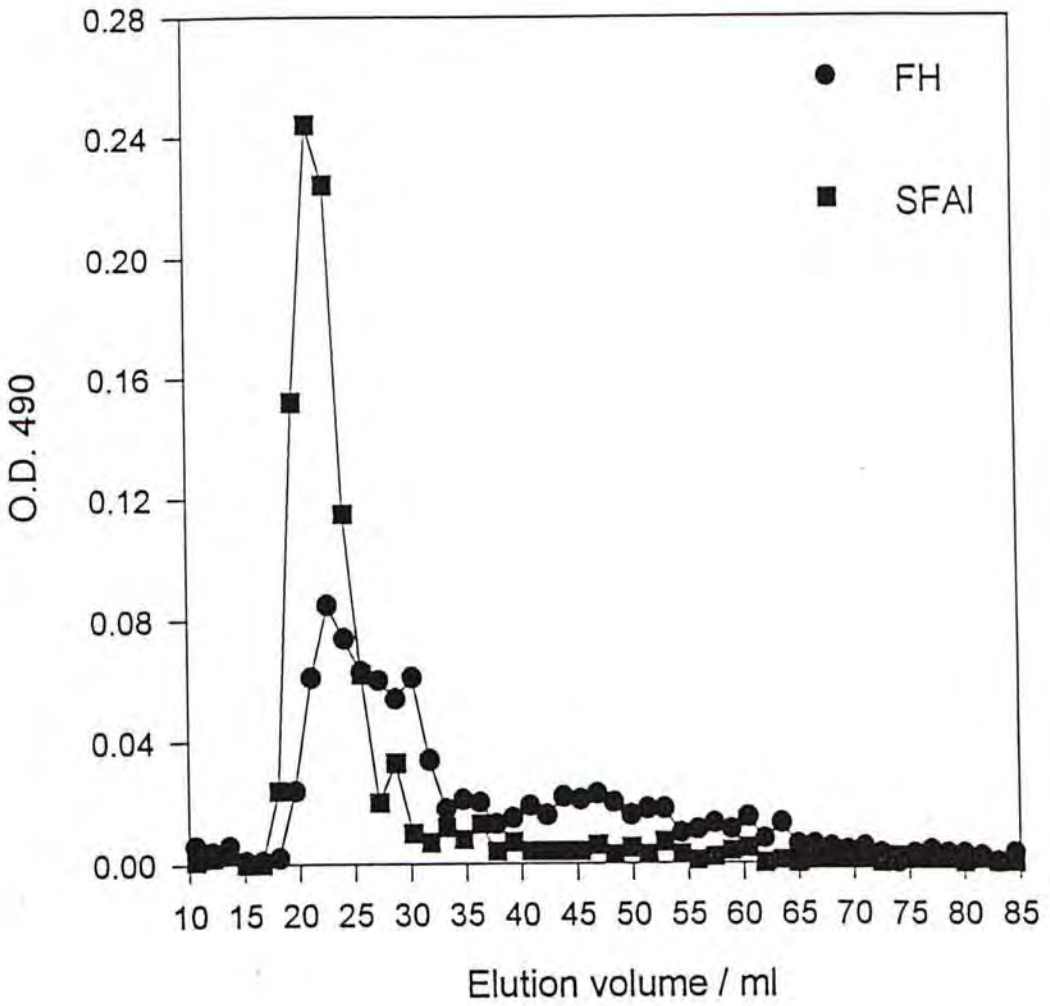


Fig. 3.11 Gel permeation (Sephadex G-200) of FH and SFAl. NaCl (0.15 N) was used as the running buffer. The flow rate of the column was adjusted to 0.5 ml/ min. with a peristaltic pump. The eluent of the column was collected by a fraction collector (1.1 ml/ tube). FH or SFAl in the eluent was monitored by phenol-sulfuric acid method and absorbance measured at 490 nm.

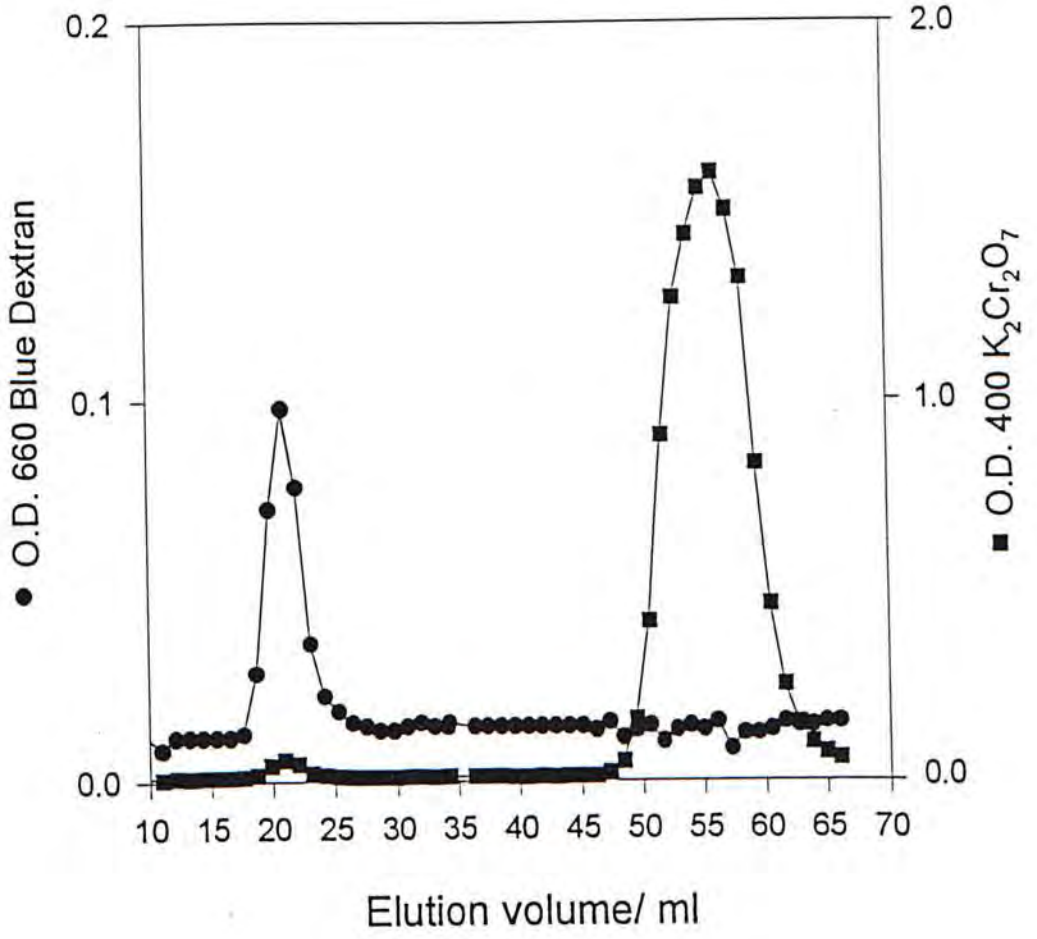


Fig. 3.12 Gel permeation (Sephadex G-100) of Blue Dextran and $K_2Cr_2O_7$. NaCl (0.15 N) was used as the running buffer. The flow rate of the column was adjusted to 0.5 ml/ min. with a peristaltic pump. The eluent of the column was collected by a fraction collector (1.1 ml/ tube). Blue Dextran in the eluent was monitored by measuring asorbance at 660 nm and $K_2Cr_2O_7$ in the eluent was monitored by measuring asorbance 400 nm.

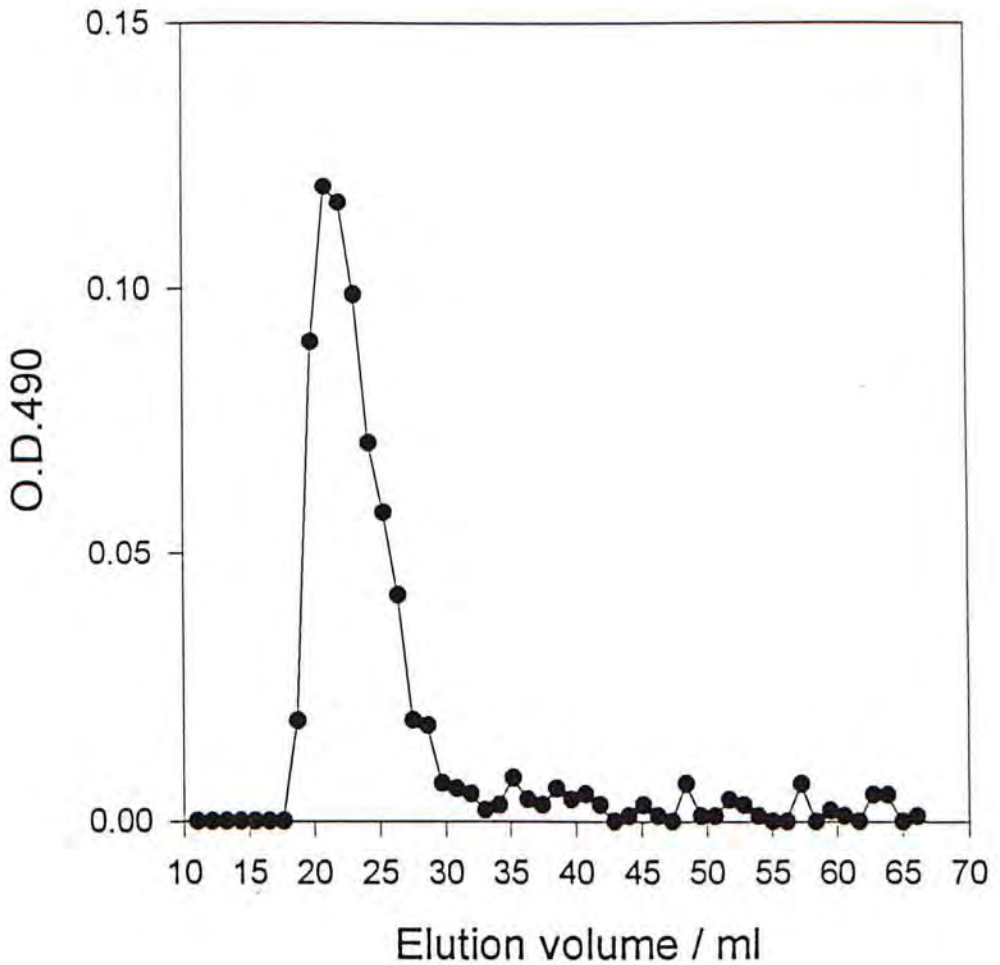


Fig. 3.13 Gel permeation (Sephadex G-100) of FH. NaCl (0.15 N) was used as the running buffer. The flow rate of the column was adjusted to 0.5 ml/ min. with a peristaltic pump. The eluent of the column was collected by a fraction collector (1.1 ml/ tube). FH in the eluent was monitored by phenol-sulfuric acid method and absorbance measured at 490 nm.

Fig. 3.15, it was found that only FH and SFAl shown VDH response in the tested period, but not IFAl. Furthermore, SFAl showed a stronger and longer lasting response when compared with FH.

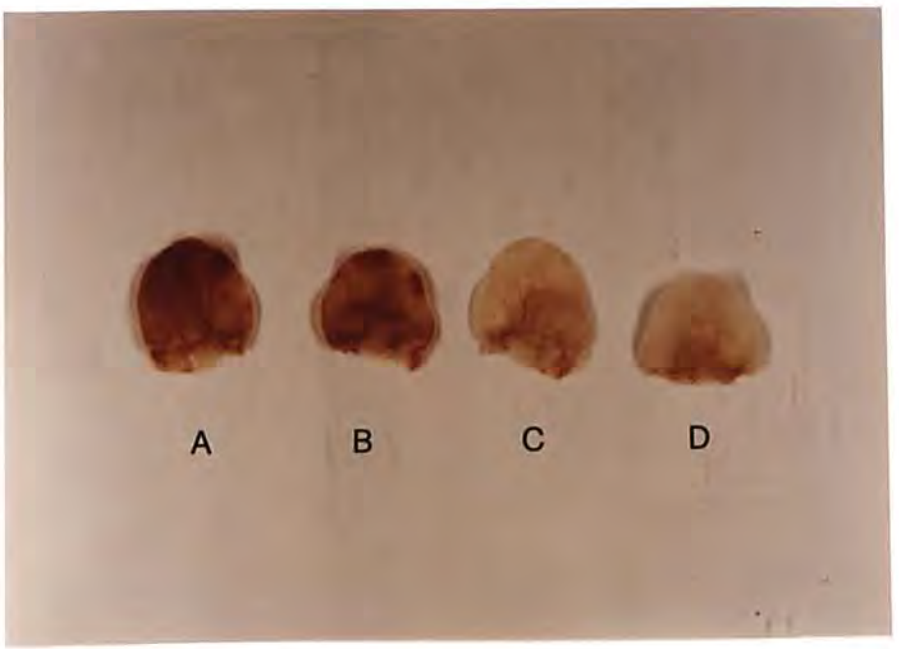


Fig. 3.14 Inflammation of male ICR albino mice induced by F.V. fractions; Vascular Dilation and Hemorrhage (VDH) response. A was ear removed from mice injected with SFAl, B was ear removed from mice injected with FH, C was ear removed from mice injected with NaCl and D was ear removed from mice injected with dextran (MW 72,200).

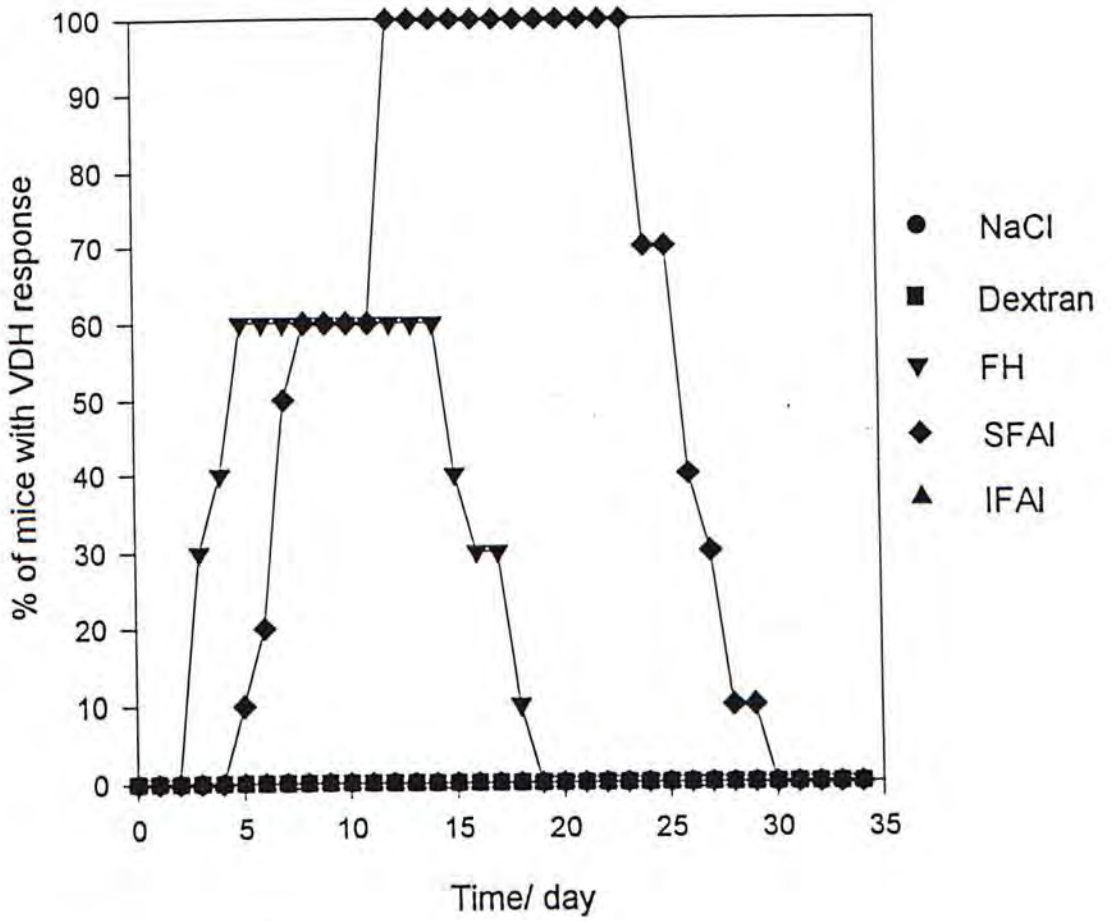


Fig. 3.15 The VDH inducing ability of F.V. fractions. Male ICR albino mice were divided into groups of ten. The mice were injected intraperitoneally with either NaCl, FH, SFAl, IFAl or dextran, for four consecutive days (from day 0 to day 3). The % of mice with VDH response was recorded everyday after the first injection until day 35.

Discussion

In the screening, all mushrooms chosen were stained positive by aniline blue, which stain β-(1→3)-D-glucan specifically (Nakanishi *et al*, 1974, 1976). However, it was found that the cell wall of *Flammulina velutipes*, was stained the most intense and was selected as a rich source of β-(1→3)-D-glucan in this project. The β-(1→3)-D-glucan, a structural component of the cell wall, was covalently attached to other insoluble wall matrix. Due to this structural arrangement, the hot water extraction process was not effective in releasing the β-(1→3)-D-glucan. Indeed, the percentage yield of FH only represent 0.5 % of the dry mushroom and most of the β-(1→3)-D-glucan present in the cell wall escaped the hot water extraction procedure. When using alkaline-urea mixture instead of water as the extraction solvent all the remaining glucan in the cell wall were released. The yield of the glucan (FAI) by this extraction method was 7 times that of the hot water extraction method. About 80 % of the FAI fraction was water soluble (the SFAI) and the remaining 20 % was water-insoluble (the IFAI).

In order to prove the identity of the fractions, the fractions were subjected to chemical and physical analysis. Since IFAI was water insoluble, analysis involved spectrophotometric measurement was not suitable for IFAI. If the extracted polysaccharides were β-(1→3)-D-glucan, they would be very likely to be a homopolymer of glucose. As expected, the carbohydrate content of all the fractions was closed to 100 %. The results from protein content and uronic acid content quite agree with that of carbohydrate content determination. In fact, the protein and uronic acid content of the fractions was insignificant—no more than 5 % of the total mass of the sample. The sugar component of the fractions was determined in order to prove whether the fractions were glucose homopolymer. In the determination, glucose, galactose, mannose, xylose, fucose and fructose were chosen as standards in the determination as these sugars were commonly found in fungal cell wall (Crook *et al*, 1961). As expected, the results of the

method showed that there was only glucose present in the hydrolysate of the fractions. When the fractions were subjected to periodate oxidation the results showed that the fractions contains linkage other than β-(1→3)-D-linkage. Periodate cleaves carbon-carbon single bond of two adjacent carbons which bear free hydroxyl groups (Goldstein *et al*, 1976; Kabat and Mayer's, 1971). The fractions, FH and SFAl, showed about 23 % of periodate uptake. If the fractions were homopolymer of glucose with β-(1→3)-D-linkage only, the periodate uptake of the fractions should be close to zero. When the percentage of periodate uptake of dextran was compared with that of the fractions the ratio of the percentage uptake was 2.00 : 0.82. Since dextran is glucose polymer linked together by α-(1→6)-D-linkage as shown in Fig. 3.16, there should be 2 cleavages by periodate per every glucose monomer in dextran. From the ratio, it was clear that there was less than one cleavage per glucose monomer in the fractions. It was likely that the periodate uptake of the fractions was due to the attachment of glucose branching to the β-(1→3)-D-glucose main chain in the form of β-(1→6)-D-linkage. The speculation base on the fact that many β-(1→3)-D-glucans isolated from different fungi do possess some β-(1→6)-D-linkages. The branch-chain may contain one to several D-glucosyl units linked at the O-6 atom such as β-D-glucans from *Phytophthora* species (Kraus *et al*, 1992; Wang *et al*, 1974), Coriolan from *Coriolus versicolor* (Miyazaki *et al* 1974; Ito *et al*, 1979), AP fraction from *Grifola frondosa* (Ohno *et al*, 1984), Scleroglucan from *Sclerotium glucanicum* (Singh *et al*, 1974), β-D-glucan from *Cochliobolus miyabeanus* (Nanba *et al*, 1987), lentinan (LNT) from *Lentinus edodes* (Chihara *et al*, 1970), schizophyllan (SPG) from *Schizophyllum commune* (Misaki *et al*, 1981) etc.. If the periodate uptake was really due to β-(1→6)-D-linked D-glucose moiety, it was estimated that there was 40 % of the glucose units in the fractions contributed to the β-(1→6)-D-linkage. The value was possible. In case of schizophyllan, lentinan and β-D-glucan from *Phytophthora* species, it was 25 %, 29 % and 60 % respectively. The structure of schizophyllan and lentinan was shown in Fig. 3.17.

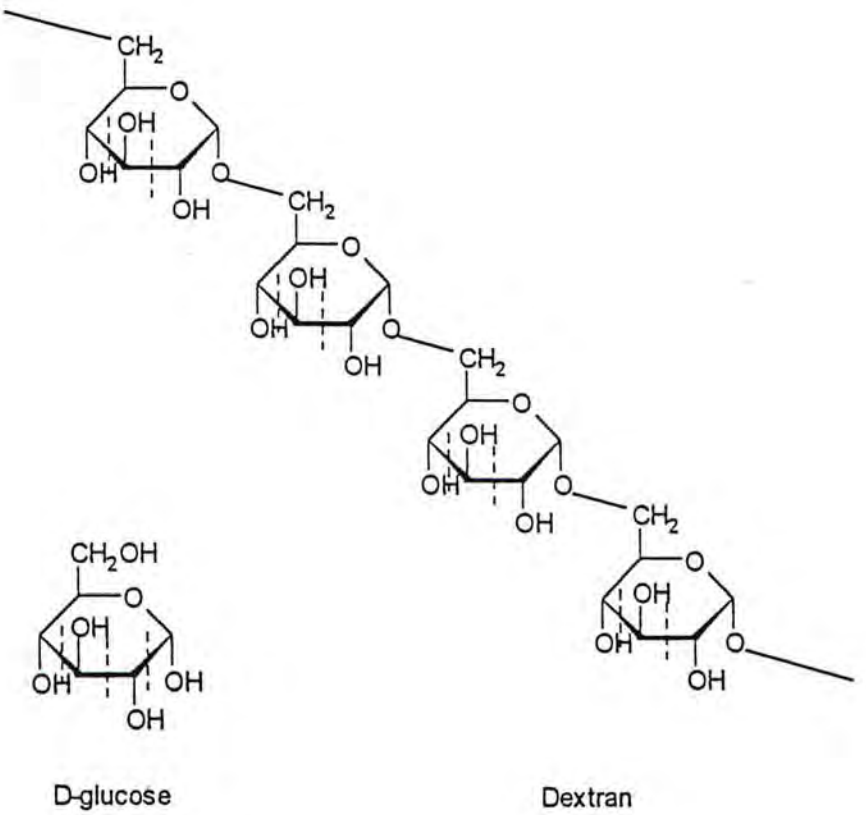
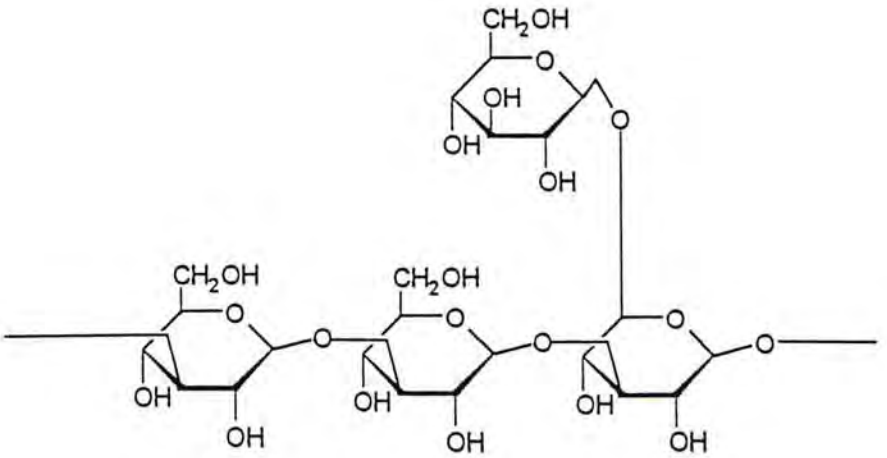
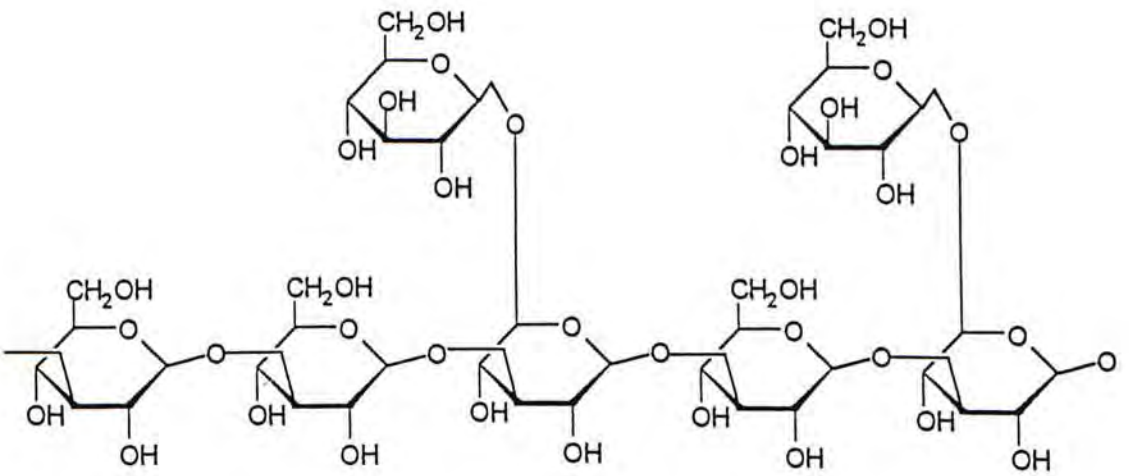


Figure 3.16 Chemical structure of D-glucose and Dextran. Dotted line represents cleavage site of periodate.



Schizophyllan (SPG)



Lentinan (LNT)

Figure 3.17 Chemical structure of Schizophyllan (SPG) and Lentinan (LNT).

All the polysaccharide fractions extracted have the capacity to coagulate the Limulus amebocyte lysate (LAL) biochemical reagent. This further support the idea that the fractions were β-(1→3)-D-glucans as the coagulation of the reagent was sensitive to β -(1→3)-D-glucan and LPS (Morita *et al*, 1981; Saito *et al*, 1991). The results from the *in vitro* mitogenic and *in vivo* TNF induction assays suggested that there existed no or insignificant amount of LPS in the fractions. In fact, the LPS contamination was estimated to be too low in the fractions to activate the LAL biochemical reagent to coagulate. When the fractions were subjected to Laminarinase enzyme , which digest β-(1→3)-D-glucan, digestion. Glucose was released from all the fractions. However, only SFAI fraction was nearly completely digested. FH showed 50 % digestion and IFAI showed about 15 % digestion. The incomplete digestion of FH may be due to the present of other polysaccharides in FH fraction or the secondary structure of FH. In later discussion, it would point out that FH and SFAI bear different secondary structure and SFAI bears the same secondary structure as the natural substrate (Laminarin) of the enzyme Laminarinase. The low digestion of the IFAI fraction may be due to the physical appearance (the IFAI fraction was water insoluble) of IFAI.

All the work done previously tried to prove the primary structure of the fractions only. To reveal the secondary structure of the fractions, IR, NMR spectrum or X-ray analysis data of the fractions should be obtained. Instead of IR, NMR and X-ray analysis, a cheaper, faster and simpler method was adopted to investigate the secondary structure—the Congo Red method (Kraus *et al*, 1992). From the results, the curve of FH was a characteristic of β-(1→3)-D-glucan with a triple helical structure, and the curve of SFAI was a characteristic of β-(1→3)-D-glucan with a single helical structure.

The fractions , if soluble in water, were highly viscous solution when dissolved in distilled water. This observation suggested that the fractions were macromolecules of very large molecular weight. The molecular weight of the fractions were estimated by gel permeation chromatography. SFAI was estimated to have molecular weight greater than

or equal to 200 kD. For FH fractions, it was treated with alkaline before applying to chromatographic separation, to reduce the viscosity of the sample. As clearly shown in the results of the Congo Red method, the alkaline condition used in the treatment broke the triple helix of FH to form single helix. Hence, the estimated value should be three times that of the determined value for FH fraction in molecular weight. The molecular weight of FH fraction was estimated to be greater than or equal to 600 kD (3× 200 kD) for the major elution peak. The minor elution peak did not appear when FH was separated by G-100 but by G-200. This suggested that the minor peak of FH should have molecular weight of greater than or equal to 300 kD (3× 100 kD).

It was reported that fungal antitumor polysaccharides with β-(1→3)-D-linkage can trigger a unique type of inflammatory response; vascular dilation and hemorrhage activities (VDH) (Aoki, 1983). There exist a high correlation between the inflammation and the anti-tumor activity of the polysaccharides. This reaction was thus used as the basis for the selection of the extracted fractions for further studies. Amount the fractions, only FH, FAl and SFAl have the capacity to trigger VDH responses and FH and SFAl were selected for further investigation.

The Toxicity of *Flammulina velutipes*

CHAPTER FOUR

THE TOXICITY OF *Flammulina velutipes*

Introduction

Most chemical agents employed in cancer therapy are highly toxic. They preferentially affect dividing cells in tumors and their hosts, and the side effects are often most apparent in those tissues or organs where there is a rapid cell turnover. These include the bone marrow, gastrointestinal epithelium and hair follicles. As a potential antitumor agents, their toxicity should be evaluated.

Results

4.1 LACK OF CYTOTOXICITY OF *Flammulina velutipes* TO BRINE SHRIMP

FH, SFAI and IFAI were first assayed for their cytotoxicity towards brine shrimp larva (*Artemia salina*). The results of the experiment were shown in Table 4.1. Cycloheximide, a protein synthesis inhibitor, was used as a positive control. The experimental value of the LC_{50} (μg/ml) of cycloheximide is about 1.6 fold larger with respect to the reported value. From the results, FH, SFAI and IFAI all shown LC_{50} (μg/ml) greater than 1000.

Table 4.1 Cytotoxicity of FH and SFAl on Brine Shrimp

Sample	LC_{50} µg/ ml (Observed Value)	LC_{50} µg/ ml (Reported Value)
Cycloheximide	47.3 ± 6.9	29.4 ± 0.3
FH	> 1000	/
FAl	> 1000	/
SFAl	> 1000	/
IFAl	> 1000	/

Suspension of nauplii, 100 µl, containing 10-25 organisms was mixed with 100 µl of test sample of various concentrations. The mixture was incubated at 26-29 °C for 24 hr. At the end of the incubation period, the number of nauplii that cannot survive the incubation was counted.

/ means that the samples do not have any reported value.

4.2 LACK OF CYTOTOXICITY OF *Flammulina velutipes* TO MURINE BONE MARROW CELLS

The cytotoxicity of FH and SFAI were further tested on mouse bone marrow cells. Three concentrations of FH and SFAI were tested (25 μg/ml, 50 μg/ml and 100 μg/ml). Cycloheximide was used as the control. As shown in Fig. 4.1, unlike cycloheximide, which caused more than 70 % decrease in DNA incorporation by the bone marrow cells, FH and SFAI caused no decrease in DNA incorporation of the bone marrow cells.

4.3 LACK OF CYTOTOXICITY OF *Flammulina velutipes* TO MOUSE

Female BALB/c mice were used in the assay. The results were shown in Table 4.2. The results showed that the injection of both FH and SFAI into mice intravenously had no lethal effect in the tested period.

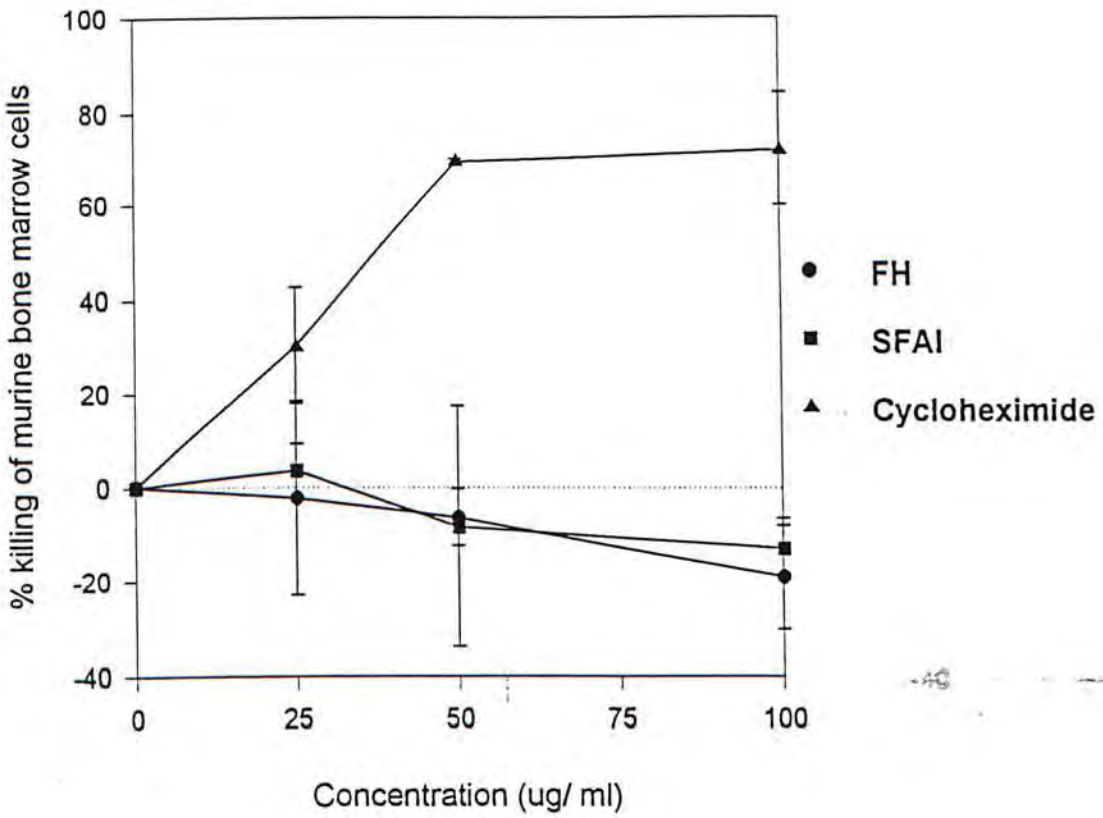


Fig. 4.1 The toxic effect of FH and SFAI on bone marrow cells of female BALB/c mice. Various concentrations (50 μg/ ml, 100 μg/ ml and 200 μg/ ml) of 100 μl FH, SFAI, or cycloheximide were cultured with 100 μl bone marrow cells (10^6 cells /ml). The cell mixtures were incubated at 37 °C with 5 % CO_2 supply for 72 hr. The killing of the bone marrow cells was monitored by thymidine uptake. Vertical bar represents one standard error.

Table 4.2 Cytotoxicity of FH and SFAl on female BALB/c mice.

Sample	LD_{50} µg/ mouse
NaCl (0.15 N)	> 1000
FH	> 1000
SFAl	> 1000

Female BALB/c mice were divided into groups of 10. Test sample (2.5 mg/ ml) or 0.15 N NaCl, 0.2 ml, was injected intravenously. The number of mice , that survive the treatment, was recorded every day after the injection until day 35 was reached.

Table 4.2 Cytotoxicity of FH and SFAl on female BALB/c mice.

Sample	LD_{50} µg/ mouse
NaCl (0.15 N)	> 1000
FH	> 1000
SFAl	> 1000

Female BALB/c mice were divided into groups of 10. Test sample (2.5 mg/ ml) or 0.15 N NaCl, 0.2 ml, was injected intravenously. The number of mice , that survive the treatment, was recorded every day after the injection until day 35 was reached.

Discussion

The toxicity of FH and SFAl were first tested with brine shrimp. The test provide a cheap, rapid, simple and reliable method (Meyer *et al*, 1982) for preliminary study. It was demonstrated that the isolated macromolecules were not toxic at the concentration tested (LC_{50} >1000 μg/ ml). As mentioned before, the most susceptible cells to the attack of chemotherapeutic drugs are rapidly dividing cells. Mouse bone marrow cells were chosen as the representative rapidly dividing mammalian cells to test for the toxic effect of FH and SFAl. Both FH and SFAl showed no significant toxic effect on the bone marrow cells *in vitro*. However, these two models failed to detect the toxic effect of chemicals which were not toxic in their native form but toxic after metabolised with liver. Even FH and SFAl were shown to be non-toxic to brine shrimp and bone marrow cells, the models failed to detect the toxic effect of compounds which show no toxicity in their native form but show toxicity after metabolisation in liver cells. For example, brine shrimp assay failed to detect the toxicity of cyclophosphamide which is known to require metabolic activation in the liver for activity in man (Solis *et al*, 1992). Whether chemicals are toxic after metabolised with liver can be partly reflected by the toxicity of the chemicals to the whole animals. FH and SFAl when injected intravenously into mice showed no significant toxic effect (LD_{50} >1000 μg/ mouse).

The Immunomodulatory Activities of *Flammulina velutipes*

CHAPTER FIVE

THE IMMUNOMODULATORY ACTIVITIES OF *Flammulina velutipes*

Introduction

The activity of the immune system, which play the centrol role in the anti-cancer mechanisms, can be modulated by many chemical agents. The modulation can be either suppression or potentiation depending on the types of chemicals involved. Drugs such as steroids and cyclophosphamide result in suppression of the immune system, and drugs such as Levamisole , Calmette-Guerin bacillus (BCG) and β-D-glucans result in potentiation of the immune system. In modern medicine, the treatment of cancer mainly rely on the use of external agents; radiation, surgical operation and cytotoxic drugs to remove or kill most of the cancer cells of a cancer patient. The complete eradication of the cancer cells still rely on the endogenous anti-cancer mechanism of the patients—the immune system. In cancer patients, the immune system of the patients is usually down regulated. Therefore, it is logical to use immunopotentiator to restore or boost up the immune system of a cancer patient in cancer treatment. B-, T-cells and macrophages are the major cells that play a role in anti-cancer mechanisms. The augmentation of the activity of these cells should help furnishing cancer cure.

Results

5.1 EFFECT OF *Flammulina velutipes* ON MURINE LYMPHOCYTES

5.1.1 Mitogenic Effect of FH and SFAI on Mouse Lymphocytes *In Vitro*

Lymphocytes obtained from female BALB/c mice were used in the assayed. FH and SFAI were cultured with the lymphocytes at various concentrations (25 μg/ml, 50 μg/ml and 100 μg/ml). The results were shown in Fig. 5.1. In the figure, FH show a gradual increase in mitogenic activity from 25 μg/ml to 100 μg/ml. At 100 μg/ml, at least 100 % increase in thymidine incorporation was observed (e.g. S.I. of 2 to 4 had been observed). However, SFAI shown no significant increase in thymidine incorporation at the concentrations tested (e.g. S.I. remains at 1). As expected, LPS and ConA caused extensive blastic transformation of the lymphocytes; e.g. the S.I. of LPS (20 μg/ml) and ConA (5 μg/ml) was 227 and 524 respectively (data not shown in the figure).

5.1.2 Mitogenic Effect of FH plus PMB on Mouse Lymphocytes *In Vitro*

It is important to check whether the mitogenic effect of FH exerted on the murine lymphocytes was due to LPS contamination. PMB, which can abolish the mitogenic activity of LPS, together with FH was added to lymphocytes culture. From the results in Fig. 5.2, the adding of PMB reduced, but not abolished, the mitogenic activity of FH. For example, the thymidine incorporation of lymphocytes cultured with FH was just slightly reduced by PMB. In case of LPS (final concentration, 10 μg/ ml), the adding of PMB (final concentration, 5 μg/ ml) reduced the thymidine incorporation, caused by LPS, by at least 50 % (data not shown in the figure).

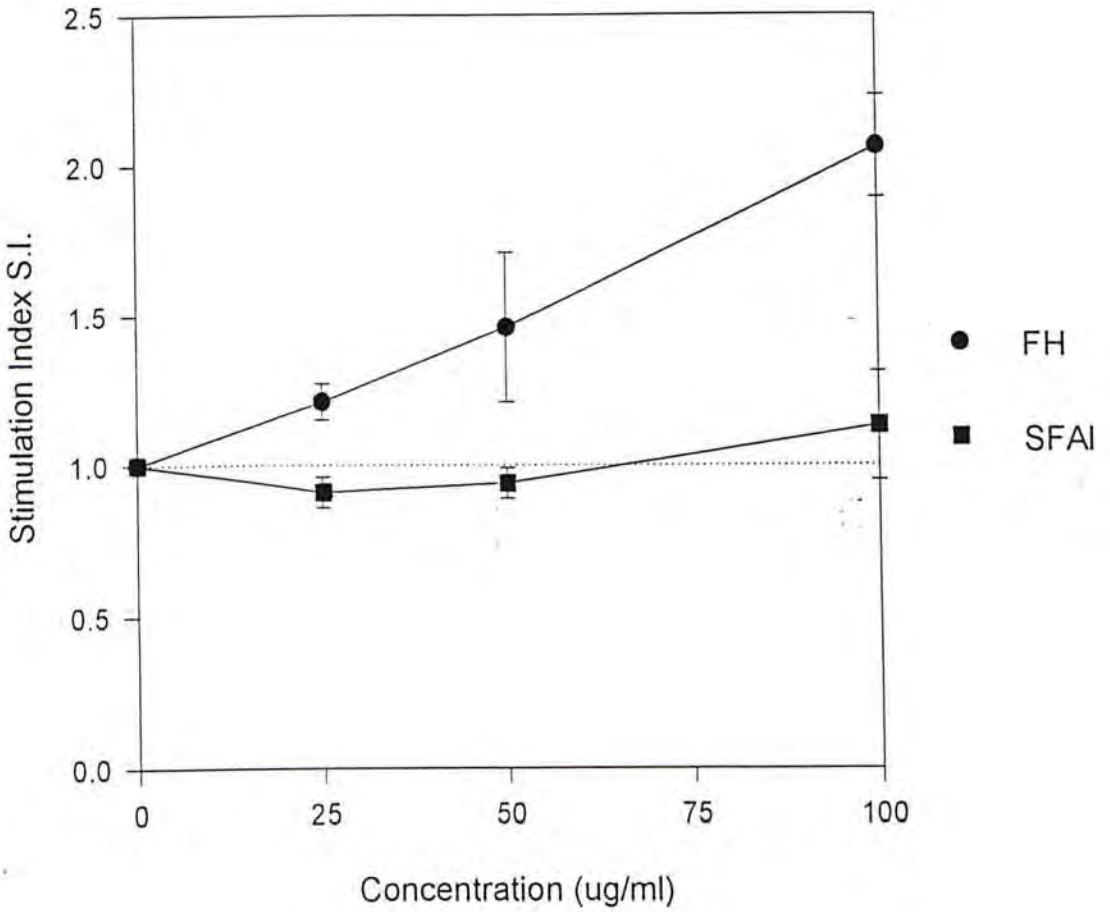


Fig. 5.1 The mitogenic effect of FH and SFAl on murine lymphocytes *in vitro*. Various concentrations of FH and SFAl were cultured with 5×10^5 mouse lymphocytes. The sample/cell mixture was incubated for 48 hr. After incubation, the cells were pulsed with 0.5 μCi ^{3}H-TdR and radioactivity incorporated was determined. Stimulation index (S.I.) is a ratio: radioactive count of sample/ radioactive count of control. Vertical bar represents one standard error.

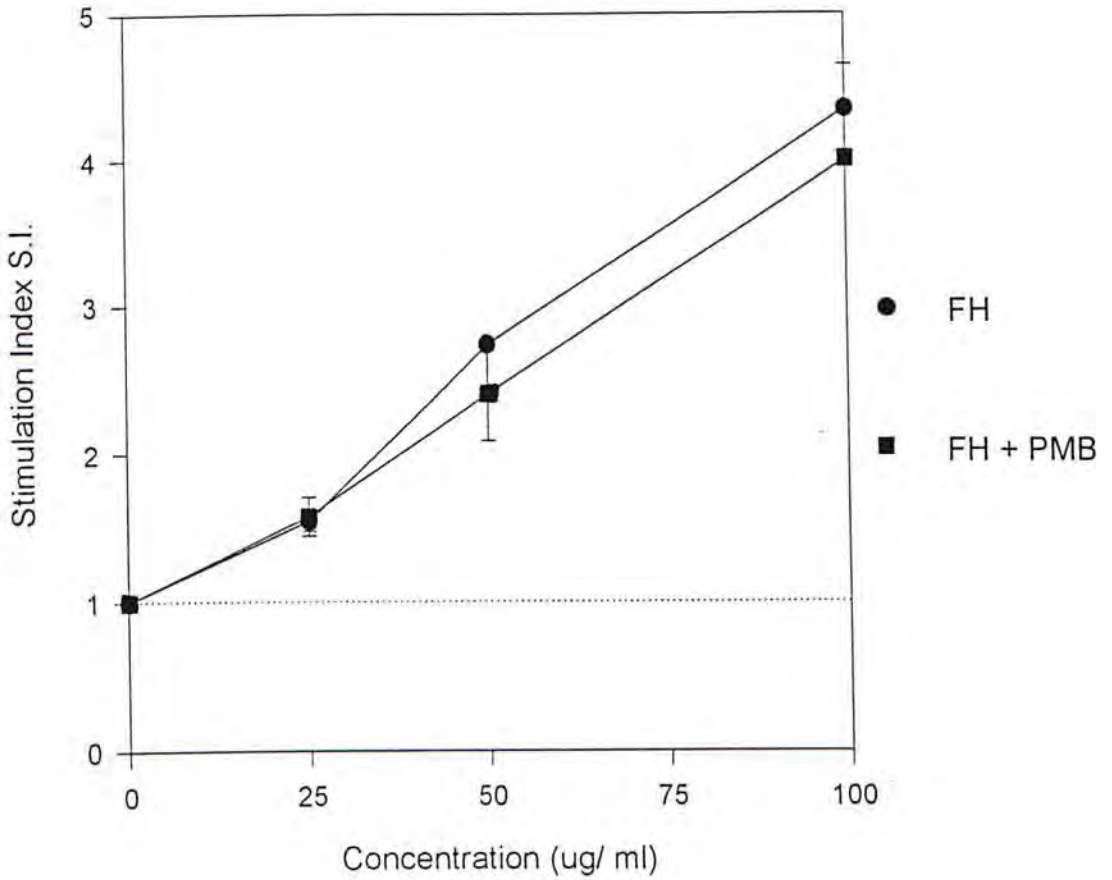


Fig. 5.2 The mitogenic effect of FH plus PMB on murine lymphocytes *in vitro*. Various concentrations of FH together with 5 μg PMB were cultured with 5×10^5 mouse lymphocytes. The sample/cell mixture was incubated for 24 hr. After the incubation, the cells were pulsed with 5 μCi ^{3}H-TdR and radioactivity was determined. Stimulation index (S.I.) is a ratio: radioactive count of sample/ radioactive count of control. Vertical bar represents one standard error.

5.1.3 Mitogenic Effect of FH on T-lymphocytes *In Vitro*

Lymphocytes obtained from female BLAB/c mice were used in the assay. T-lymphocyte subpopulation was prepared by Cederlane Column kit (an affinity column). The results were shown in Fig. 5.3. When the cell preparation was cultured with FH (100 μg/ml), ConA (1 μg/ml) or LPS (10 μg/ml) the stimulation index was 1.6, 2.8 and 70.5 respectively.

5.1.4 Mitogenic Effect of FH on B-lymphocytes *In Vitro*

Lymphocytes obtained from female BALB/c mice were used in the assay. B-lymphocyte subpopulation was prepared by adding anti-T lymphocyte antibody and complement to the lymphocyte preparation. The antibody together with the complement lysed the T-lymphocytes and remained behind the B-lymphocyte population. The B-lymphocytes were cultured with either FH (100 μg/ml), ConA (1 μg/ml) or LPS (10 μg/ml). The results were shown in Fig. 5.4. The B-lymphocytes cultured with ConA showed no significant increase in thymidine incorporation. However, B-lymphocytes cultured with FH showed an increase in thymidine incorporation; S.I. equaled 7.2. As expected, the B-lymphocytes cultured with LPS showed a great increase in thymidine incorporation; S.I. equaled 61.3.

5.1.5 Co-mitogenic Effect of FH and SFAl on Murine Lymphocytes *In Vitro*

Even SFAl cannot induce lymphocyte proliferation *in vitro*, it's co-mitogenic activity was also tested. Lymphocytes obtained from female BLAB/c mice were used in the assay. The results were shown in Fig. 5.5. Lymphocyte preparation when cultured with FH-LPS mixture and FH-ConA mixture had S.I. similar to that of lymphocytes

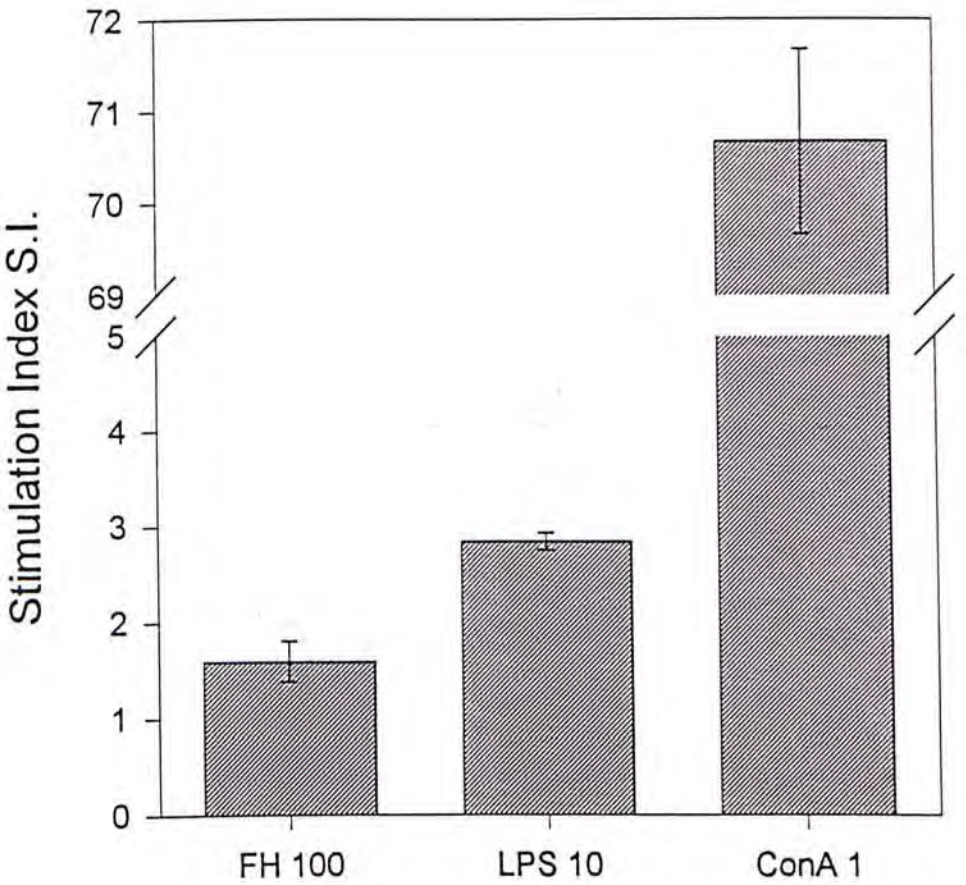


Fig. 5.3 The mitogenic effect of FH on murine T-lymphocytes *in vitro*. FH was cultured with 5×10^5 murine B-lymphocytes at 100 μg/ ml. The sample/cell mixture was incubated for 48 hr. After incubation, the cells were pulsed with 0.5 μCi ^{3}H-TdR and radioactivity incorporated was determined. Stimulation index (S.I.) is a ratio: radioactive count of sample/ radioactive count of control. Vertical bar represents one standard error.

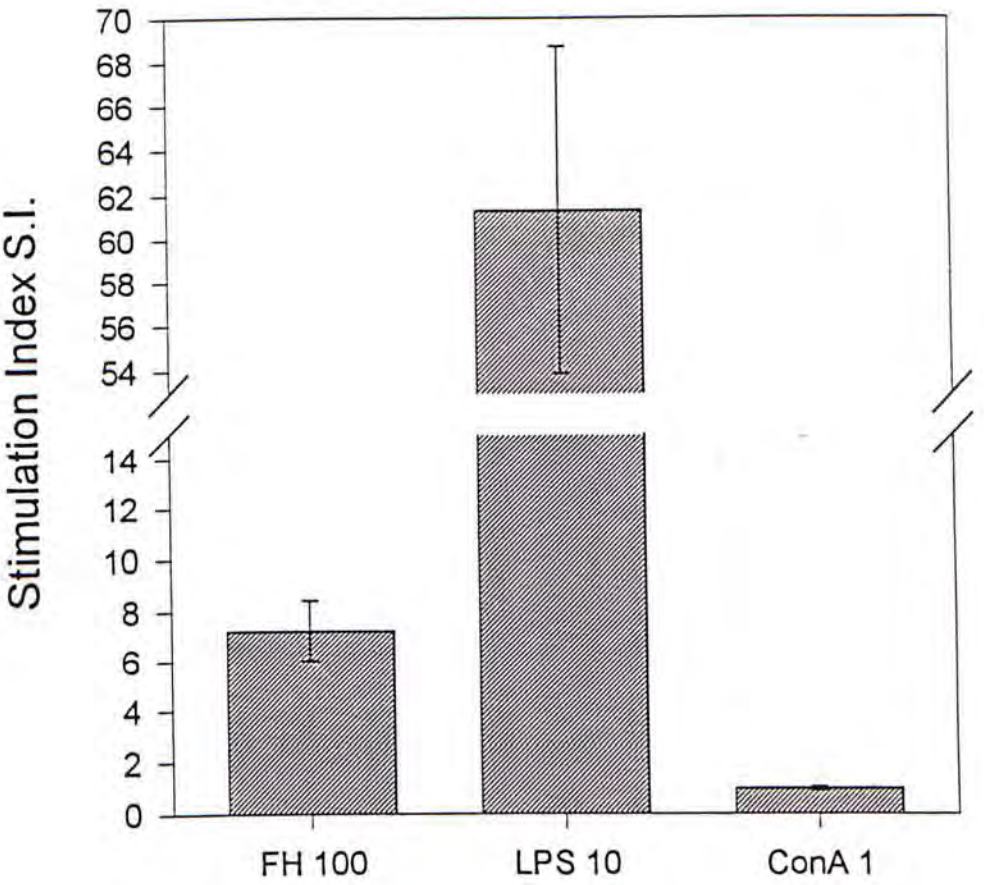


Fig. 5.4 The mitogenic effect of FH on murine B-lymphocytes *in vitro*. FH was cultured with 5×10^5 murine T-lymphocytes at 100 μg/ ml. The sample/cell mixture was incubated for 24 hr. After the incubation, the cells were pulsed with 5 μCi ^{3}H-TdR and radioactivity was determined. Stimulation index (S.I.) is a ratio: radioactive count of sample/ radioactive count of control. Vertical bars represents one standard error.

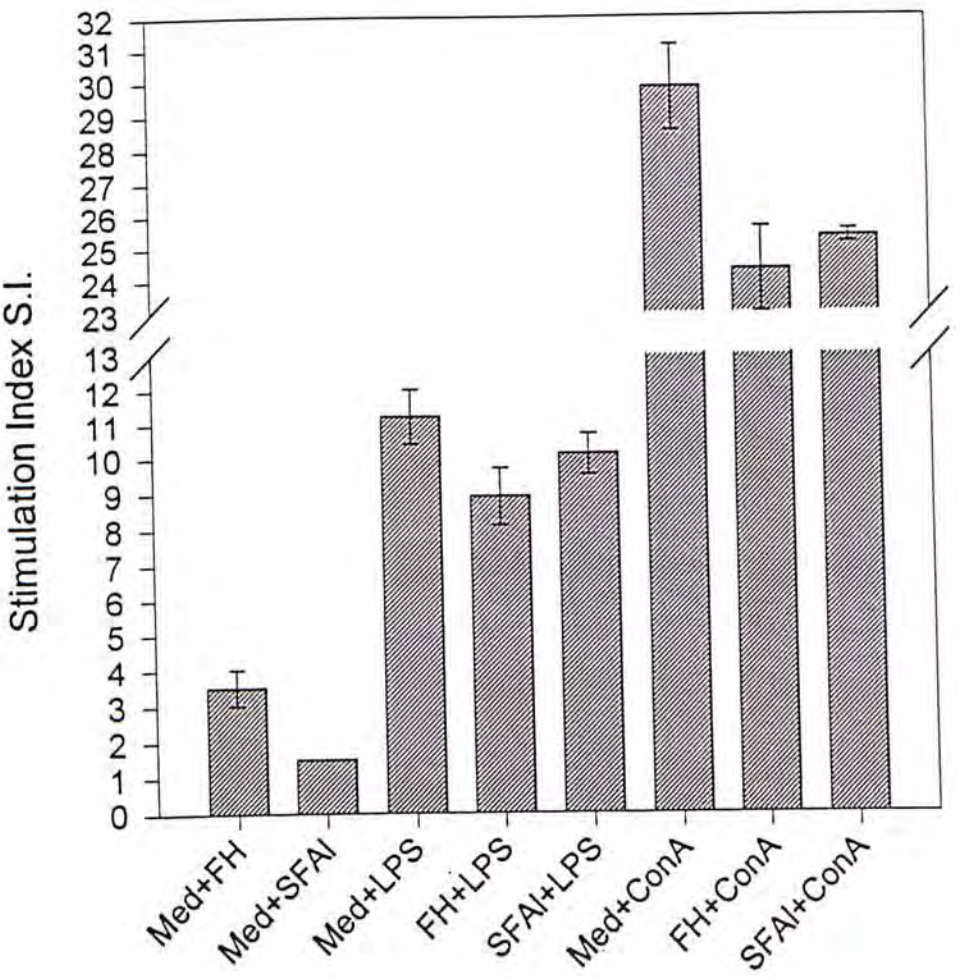


Fig. 5.5 The co-mitogenic effect of FH and SFAI on murine lymphocytes. FH and SFAI were cultured with 5×10^5 mouse lymphocytes at 100 μg/ ml. The sample/cell mixture was incubated for 48 hr. After incubation, the cells were pulsed with 0.5 μCi ^{3}H-TdR and radioactivity incorporated was determined. Stimulation index (S.I.) is a ratio: radioactive count of sample/ radioactive count of control. Vertical bar represents one standard error.

Med— Complete medium (RPMI with 10 % FCS supplemented).

cultured with LPS and ConA alone. Like FH, SFAl cannot enhance the blastic transformation ability of LPS and ConA at the concentration tested.

5.1.6 Mitogenic Effect of FH and SFAl on Murine Bone Marrow Cells *In Vitro*

Bone Marrow cells obtained from female BALB/c mice were used in the assay. As shown in Fig. 5.6, FH and SFAl tested at various concentrations (25 μg/ml, 50 μg/ml and 100 μg/ml) cannot induce the proliferation of bone marrow cells. As expected, the positive control, murine rIL-3 (200 μg/ml) induced extensive proliferation of murine bone marrow cells ; S.I. equaled 31.4 (data not shown in the figure).

5.1.7 Mitogenic Effect of FH and SFAl on Murine Lymphocytes *In Vivo*

Female C57BL/6J mice were used in the assay. The mice were injected either with NaCl, FH or SFAl on day 0. Lymphocytes were obtained from the mice on day 0, 3 and 5. The proliferation of the isolated lymphocytes were monitored by thymidine incorporation. As clearly shown in Fig. 5.7, only lymphocytes prepared from mice, 5 days after FH and SFAl injection, had significant increase in DNA synthesis; FH increased the DNA synthesis by 143 % and SFAl increased the DNA synthesis by 195 %. Lymphocytes prepared from mice, 0 and 3 days after FH and SFAl injection, had no significant increase in DNA synthesis with respect to control.

5.1.8 Effect of FH and SFAl on the Enhancement of First Antibody Production of SRBC Immunised Mice

Male ICR albino mice were injected with SRBC intraperitoneally on day 0. The mice were also injected either with NaCl, FH or SFAl on day -2, 0 and 2. Serum obtained from the mice on day 6 was tested for anti-SRBC antibody titre. As shown in

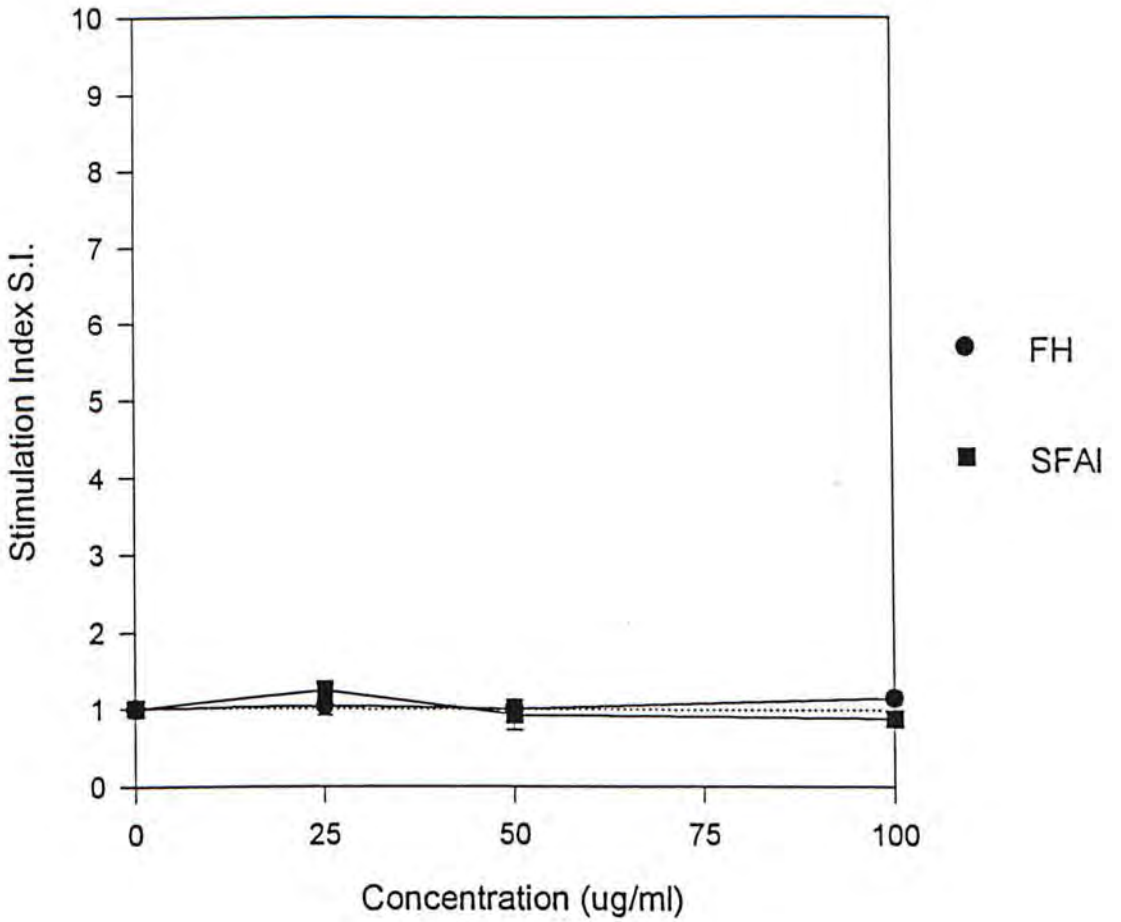


Fig. 5.6 The mitogenic of FH and SFAI on murine bone marrow cells *in vitro*. Various concentrations of FH and SFAI were cultured with 10^5 mouse bone marrow cells. The sample/cell mixture was incubated for 72 hr. After incubation, the cells were pulsed with 5 μCi ^{3}HTdR and radioactivity incorporated was determined. Stimulation index (S.I.) is a ratio: radioactive count of sample/ radioactive count of control. Vertical bar represents one standard error.

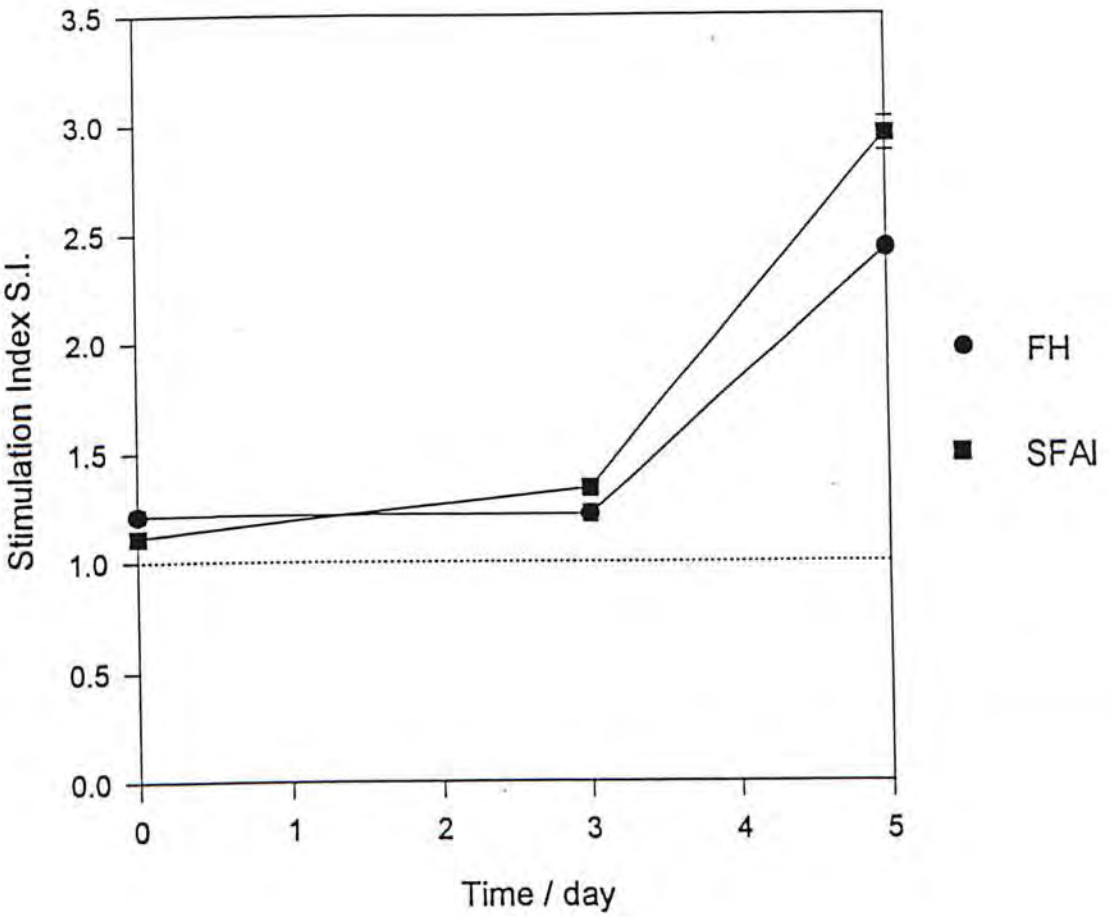


Fig. 5.7 The mitogenic effect of FH and SFAl on murine lymphocytes *in vivo*. Female C57BL/6J mice were either injected intraperitoneally with NaCl, FH or SFAl. Lymphocytes were prepared from the mice on day 0, 3 and 5. 5×10^5 of the isolated lymphocytes were pulsed with 5 μCi ^{3}H-TdR for 6 hr. and radioactivity incorporated was determined. Stimulation index (S.I.) is a ratio: radioactive count of sample/ radioactive count of control. Vertical bar represents one standard error.

Table 5.1, the range of the serum antibody titre of mice treated with FH or SFAI were roughly the same when compared with control.

5.2 EFFECT OF *Flammulina velutipes* ON MURINE MACRO PHAGE

5.2.1 Effect of FH and SFAI on *In Vitro* Phagocytic activity of Murine Macrophage

Peritoneal macrophages prepared from female BALB/c mice were used in the assay. FH and FAI at various concentrations (3.5 μg/ml, 7.0 μg/ml, 14 μg/ml and 28 μg/ml) were added to the peritoneal macrophage. The results are shown in Fig. 5.8 and Fig. 5.9, FH at the concentration tested cannot enhance macrophage phagocytic activity. However, FAI at the concentration tested increased the phagocytic activity of macrophage significantly and the activity peaked at 14 μg/ ml. In order to find out whether the activation of macrophage by FAI was due to SFAI or IFAI. SFAI or IFAI were added to peritoneal macrophage (14 μg/ml). As shown in Fig. 5.10, SFAI did not enhance the phagocytic activity of peritoneal macrophage but IFAI did.

5.2.2 Effect of FH, SFAI and IFAI on *In Vivo* Phagocytic Activity of Murine Macrophage

Female BALB/c mice were used in the assay. Three days after intraperitoneal injection with either NaCl, FH, SFAI or IFAI, peritoneal macrophages from the mice were prepared and tested for phagocytic activity. As shown in Fig. 5.11, FH enhanced the phagocytic activity slightly but FAI and SFAI enhanced the phagocytic activity markedly.

Table 5.1 Effect of FH and SFAl on the augmentation of primary antibody response of mice to SRBC.

Treatment	Antibody titre (dilution fold)
NaCl	2^6 - 2^9
FH	2^7 - 2^8
SFAl	2^7 - 2^9

Male ICR albino mice were injected with SRBC intraperitoneally on day 0. The mice were also injected either with NaCl, FH or SFAl on day -2, 0 and 2. Serum obtained from the mice on day 6 was tested for anti-SRBC antibody titre. The range of the serum antibody titre of mice treated with FH or SFAl were roughly the same when compared with control. The range was obtained from 3 separate experimental trials.

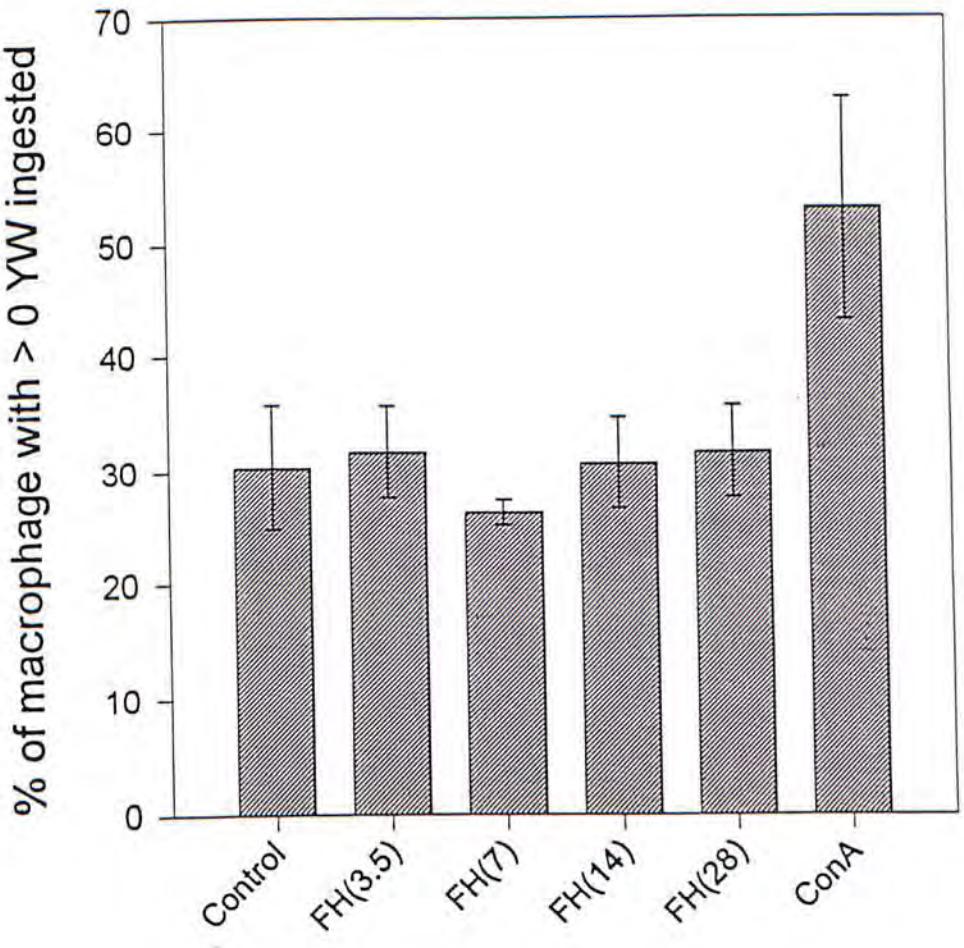


Fig. 5.8 The effect of FH on *in vitro* phagocytic activity of murine macrophages. Mouse peritoneal macrophages were treated with FH of various concentrations. After treatment, 25×10^6 yeast cell wall particles (YW) were added to the macrophages. YW ingested by macrophages was stained and the % of 100 macrophages with ≥ 1 YW ingested was counted. Vertical bar represents one standard error.

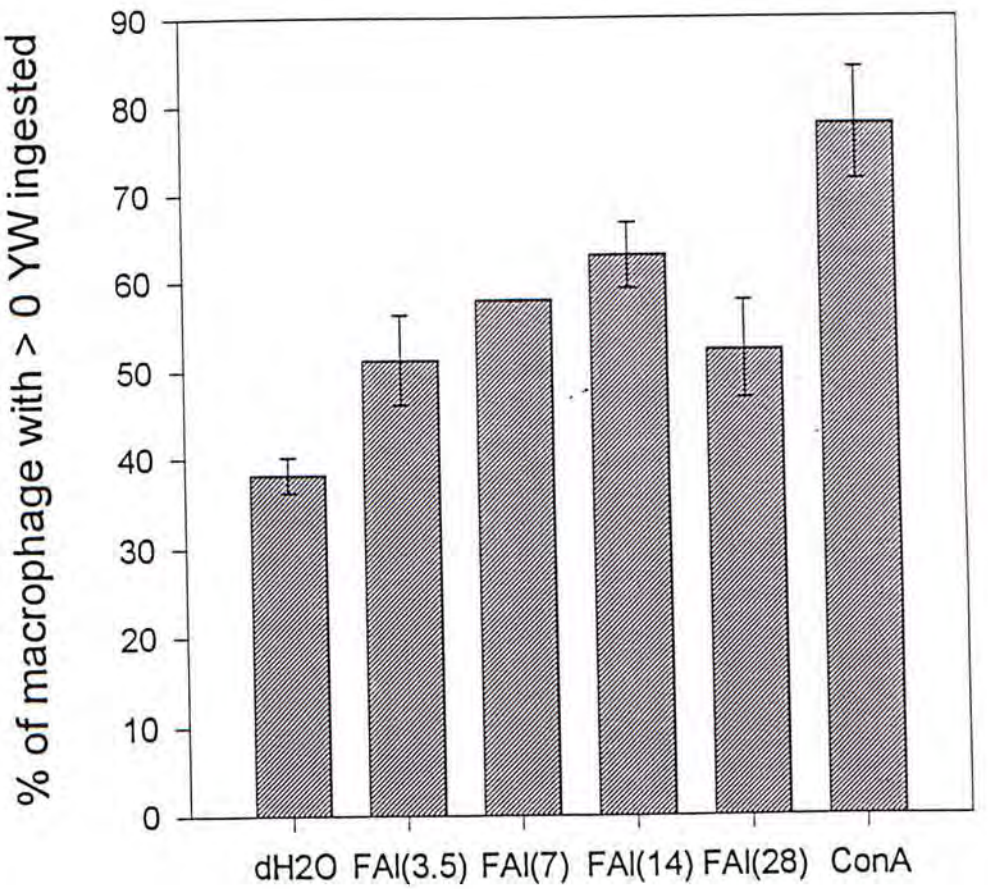


Fig. 5.9 The effect of FAI on *in vitro* phagocytic activity of murine macrophages. Mouse peritoneal macrophages were treated with FAI of various concentrations. After treatment, 25×10^6 yeast cell wall particles (YW) were added to the macrophages. YW ingested by macrophages was stained and the % of 100 macrophages with ≥ 1 YW ingested was counted. Vertical bar represents one standard error.

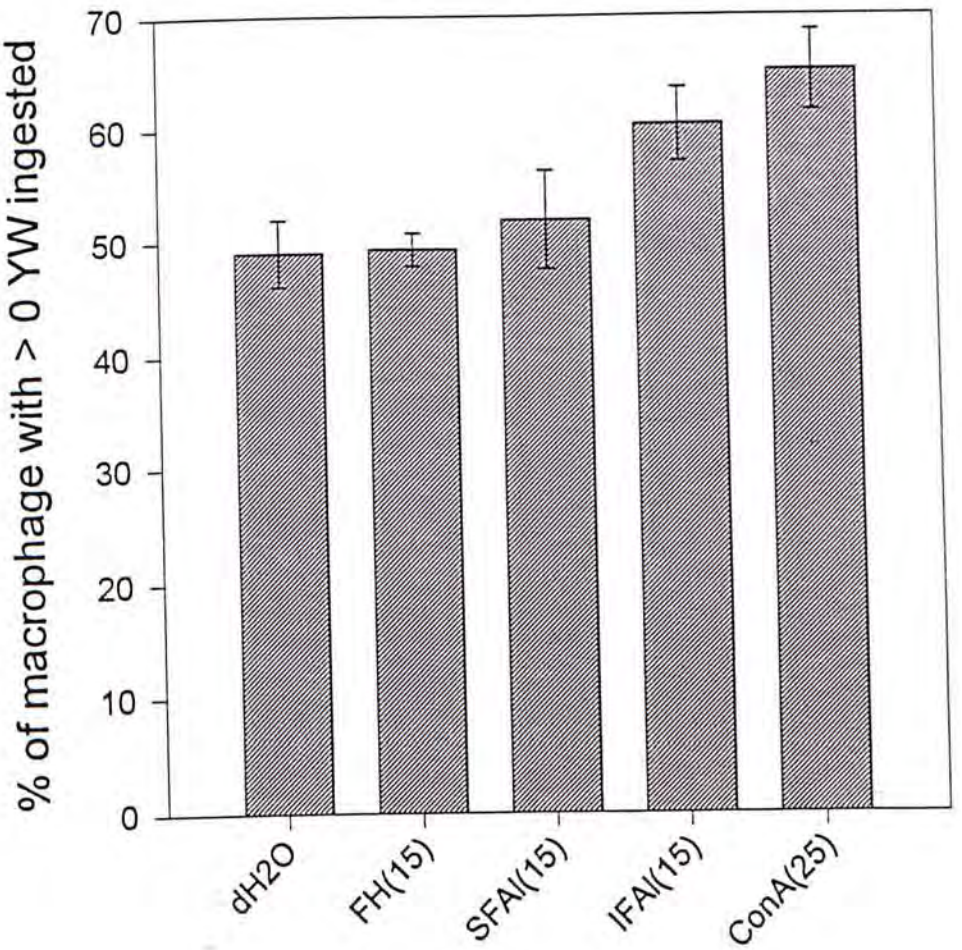


Fig. 5.10 The effect of FH, SFAI and IFAI on *in vitro* phagocytic activity of murine macrophages. Mouse peritoneal macrophages were treated with FH, SFAI or IFAI at 15 μg/ ml. After treatment, 25×10^6 yeast cell wall particles (YW) were added to the macrophages. YW ingested by macrophages was stained and the % of 100 macrophages with ≥ 1 YW ingested was counted. Vertical bar represents one standard error.

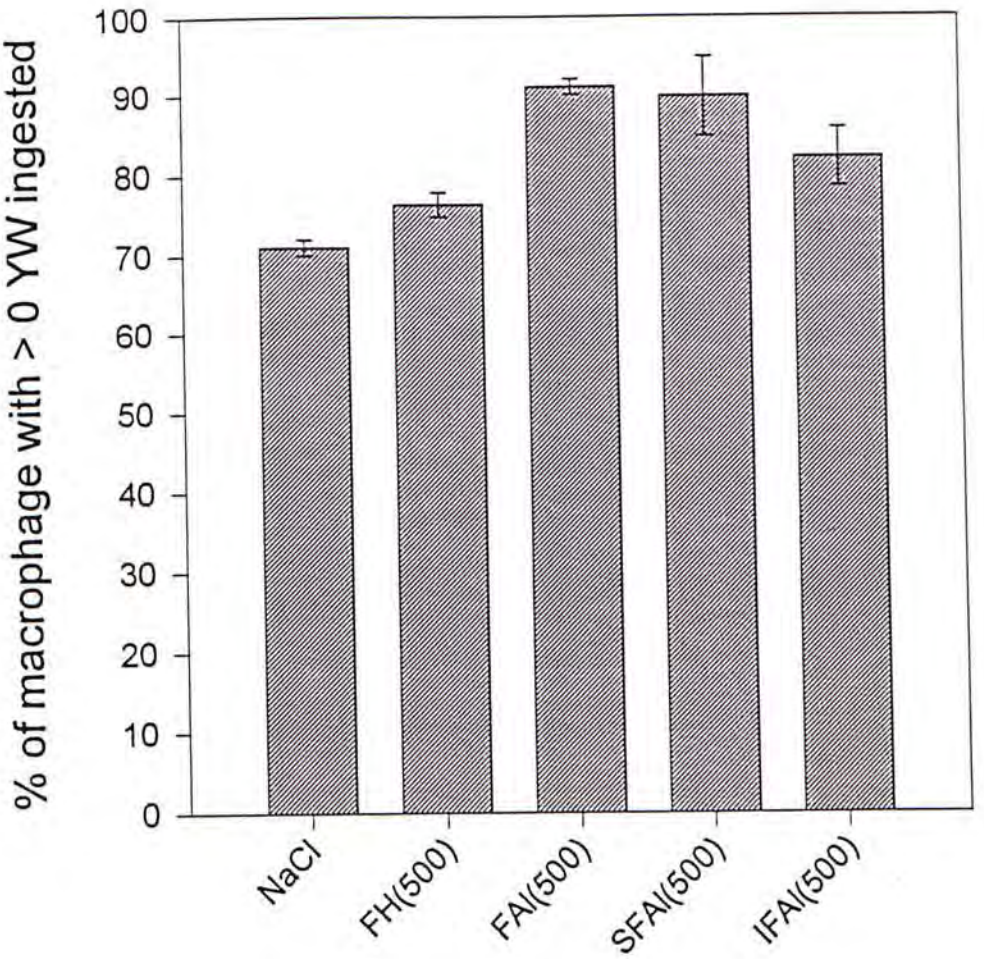


Fig. 5.11 The effect of FH, SFAl and IFAl on *in vivo* phagocytic activity of murine macrophages. Female BALB/c mice were injected either with NaCl, FH, FAl, SFAl or IFAl. Three days after treatment, peritoneal macrophages were prepared from the mice and 25×10^6 yeast cell wall particles (YW) were added to the macrophages. YW ingested by macrophages was stained and the % of 100 macrophages with ≥ 1 YW ingested was counted. Vertical bar represents one standard error.

5.2.3 Effect of FH and SFAl on *In Vivo* Migration of Murine Macrophages

Female BALB/c mice were used in the assay. Three days after intraperitoneal injection with either NaCl, FH or SFAl, peritoneal exudate cells from the mice were prepared. The macrophage population in the PEC preparation was determined by neutral red dye. As shown in Fig. 5.12, the injection of FH increased the number of macrophage by 90 % and the injection of SFAl increased the number of macrophage by 160 %. In case of thioglycollate (a positive control), the increase was about 200 %.

5.2.4 Cytostatic Activity of Macrophages in FH- and SFAl- treated mice

Female BLAB/c mice were injected with either NaCl, FH or SFAl on day 0. Three days after injection, PEC were harvested from the mice. The harvested PEC were co-cultured with L-929 tumor cells at cell ratio; 5:1, 10:1, 20:1 and 40:1 respectively. The results are shown in Fig. 5.13. As clearly shown in the figure, the percentage of tumor cell killing by PEC obtained from FH- and SFAl-treated mice had no significant increase with respect to control at cell ratio 5:1 and 10:1. However, when cell number ratio increased to 20:1 and 40:1 the percentage of tumor cell suppression by PEC obtained from FH- and SFAl-treated mice had significant increase with respect to control.

5.2.5 Effect of FH and SFAl on Fc Receptor Expression of Peritoneal Exudate Cells

Female BALB/c mice were injected intraperitoneally with either NaCl, FH or SFAl on day 0. Three days after injection, the PEC were harvested and assayed for the expression of Fc receptor. The results are shown in Fig. 5.14. In case of PEC obtained from FH treated mice, there was a gradual increase in Fc receptor expression as the

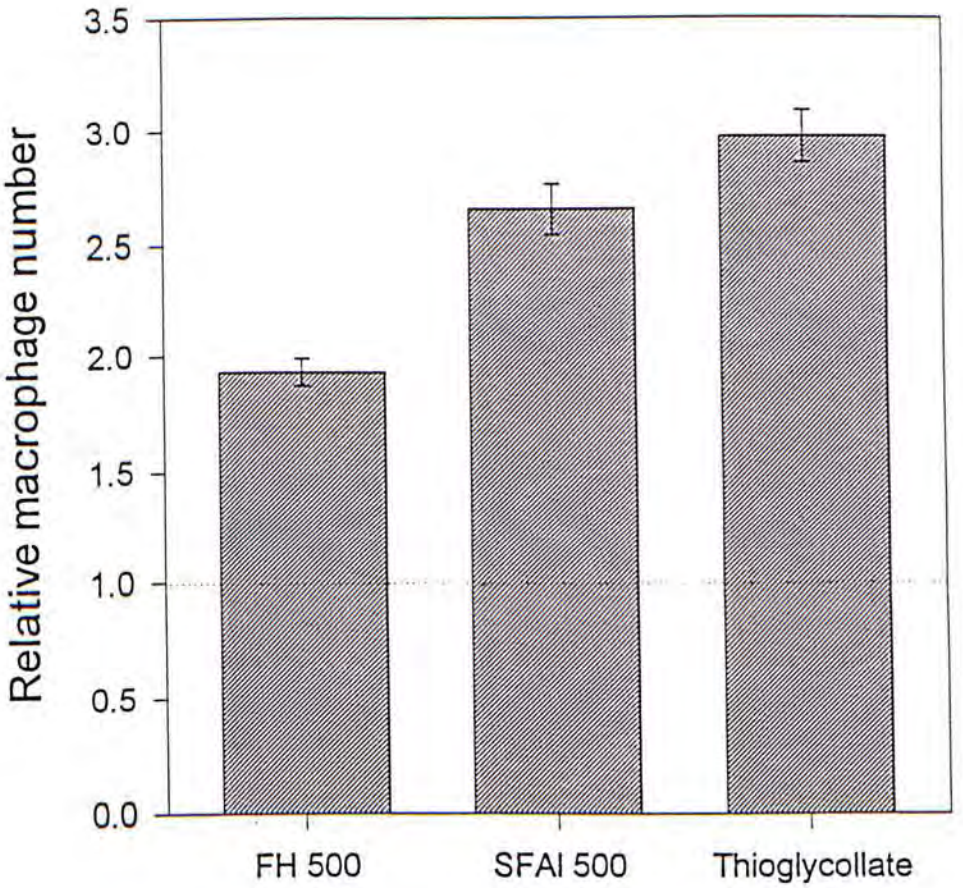


Fig. 5.12 The effect of FH and SFAl on *in vivo* migration of murine macrophages. Mice were either injected intraperitoneally with NaCl, FH or SFAl at 500 μg/ mouse. Three days after treatment, PEC were obtained from the mice. Two ml (10^6 cells/ ml) PEC suspension was added per well of a 24-well plate. The cells were incubated at 37°C for 4 hr. After incubation, non-adherent cells were washed away with PBS. The number of adherent cells was determined with neutral red dye and absorbance measured at 540 nm. The relative macrophage number was obtained from the ratio: O.D. of sample/O.D. of control. Vertical bar represents one standard error.

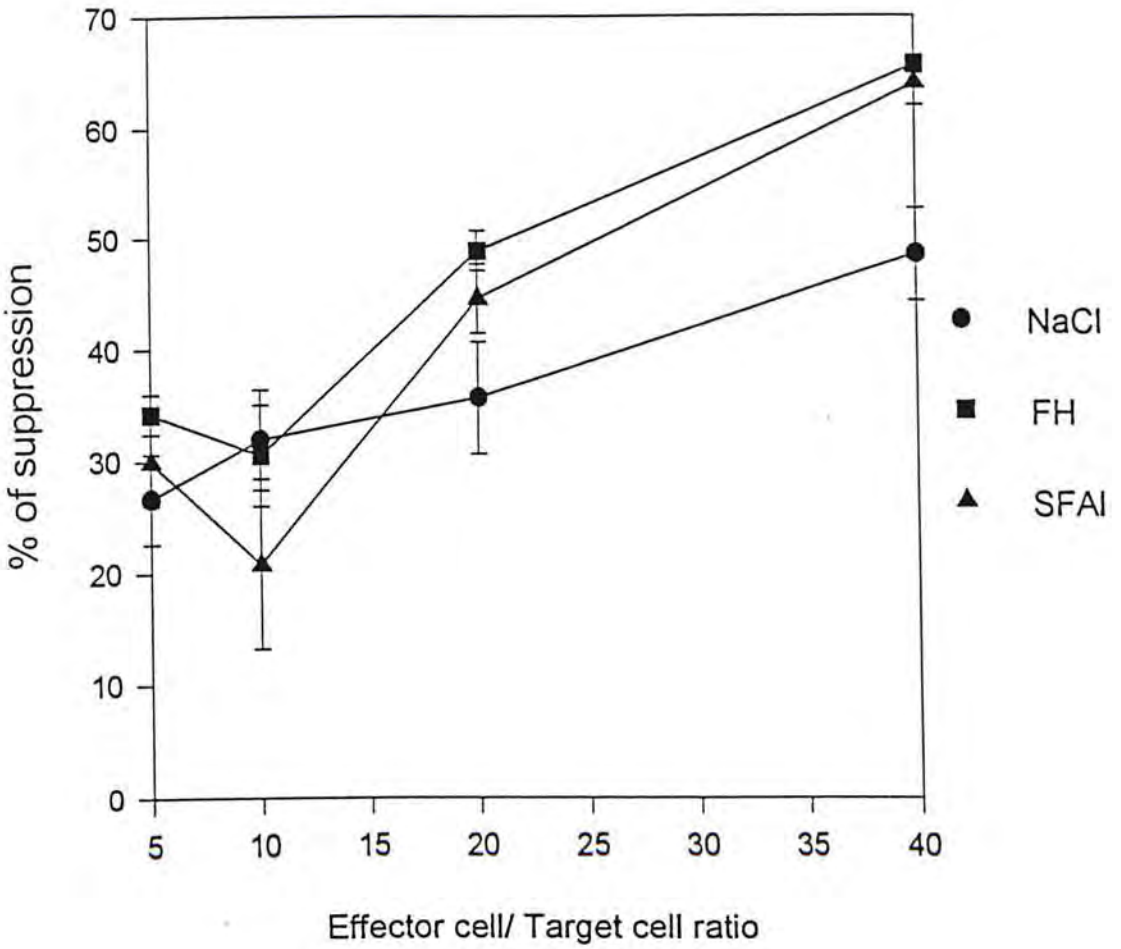


Fig. 5.13 The effect of FH and SFAl on cytostatic action of murine macrophages. Mice were injected intraperitoneally with either NaCl, FH or SFAl. Three days after injection, PEC were obtained from the mice. The prepared PEC was co-cultured with 8×10^5 L-929 cells in a 96-well plate at 40:1, 20:1, 10:1 and 5:1 cell ratio. After 4 hr. of incubation, the cell mixture was pulsed with 5 μCi ^{3}H-TdR for 6 hr. and the radioactivity incorporated was determined. Vertical bar represents one standard error.

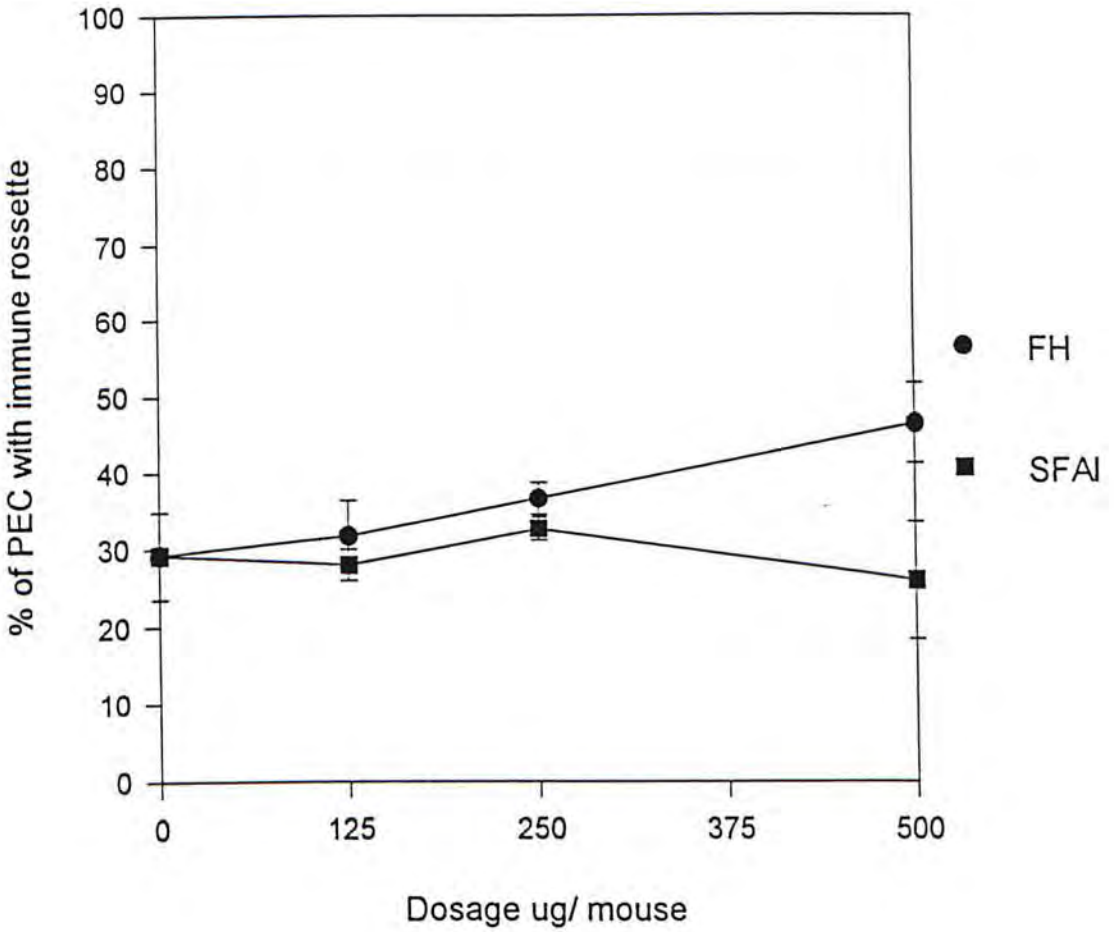


Fig. 5.14 The effect of FH and SFAI on Fc receptor expression of mouse PEC. Mice were injected intraperitoneally with either NaCl, FH or SFAI. Three days after injection, PEC were obtained from the mice. 2×10^6 PEC was mixed with 1.2×10^8 anti-SRBC antibody coated SRBC. After incubation at 37 ^oC for 15 min., cells were spun down and pellet obtained was then resuspended by gentle shaking. The number of 100 PEC with immune rossetting was counted. Vertical bar represents one standard error.

injection dosage increase from 125 μg/mouse to 500 μg/mouse. At 500 μg/mouse, the increase was significant with respect to control. In case of SFAl, there was no significant increase in Fc receptor expression at the dosage tested.

5.3 EFFECT OF *Flammulina velutipes* ON MURINE SERUM CYTOKINE AND COMPLEMENT LEVEL

5.3.1 Effect of FH and SFAl on Murine Serum Cytokine Level

Male ICR albino mice were injected intraperitoneally with either NaCl, FH or SFAl on day 0, 1, 2 and 3. Serum was obtained from the mice on day 1, day 6, day 9 and day 12. The serum GM-CSF, IL-1 α, IL-4 and INF-γ level was assayed with ELISA kits. The results are shown in Fig. 5.15, 5.16, 5.17 and 5.18 respectively. As shown in Fig. 5.15, 5.16 and 5.17, the injection of FH and SFAl induced no significant increase of serum GM-CSF, IL-1 α and IL-4. However, the injection of FH or SFAl did induce a significant increase in serum INF-γ level as shown in Fig. 5.18. SFAl when compared with FH can induce much more INF-γ release. The release peaked on day 6 and returned to basal level on day 12. To assay the effect of FH and SFAl on the induction of TNF release, female BALB/c mice were intravenously injected with either NaCl, FH, SFAl or LPS. One hour after injection, serum was obtained from the mice . The serum was diluted to different concentrations, 10 fold to 10×10^{15} fold. The diluted serum was cultured with L-929 tumor cells. The degree of L-929 cell killing was monitored by neutral red dye. The results are shown in Fig. 5.19. As shown in the figure, the TNF titre of FH- and LPS- treated, but not SFAl -treated mice, were significantly higher than that of the control. The TNS titre of FH-treated mice when compared with that of LPS was similar.

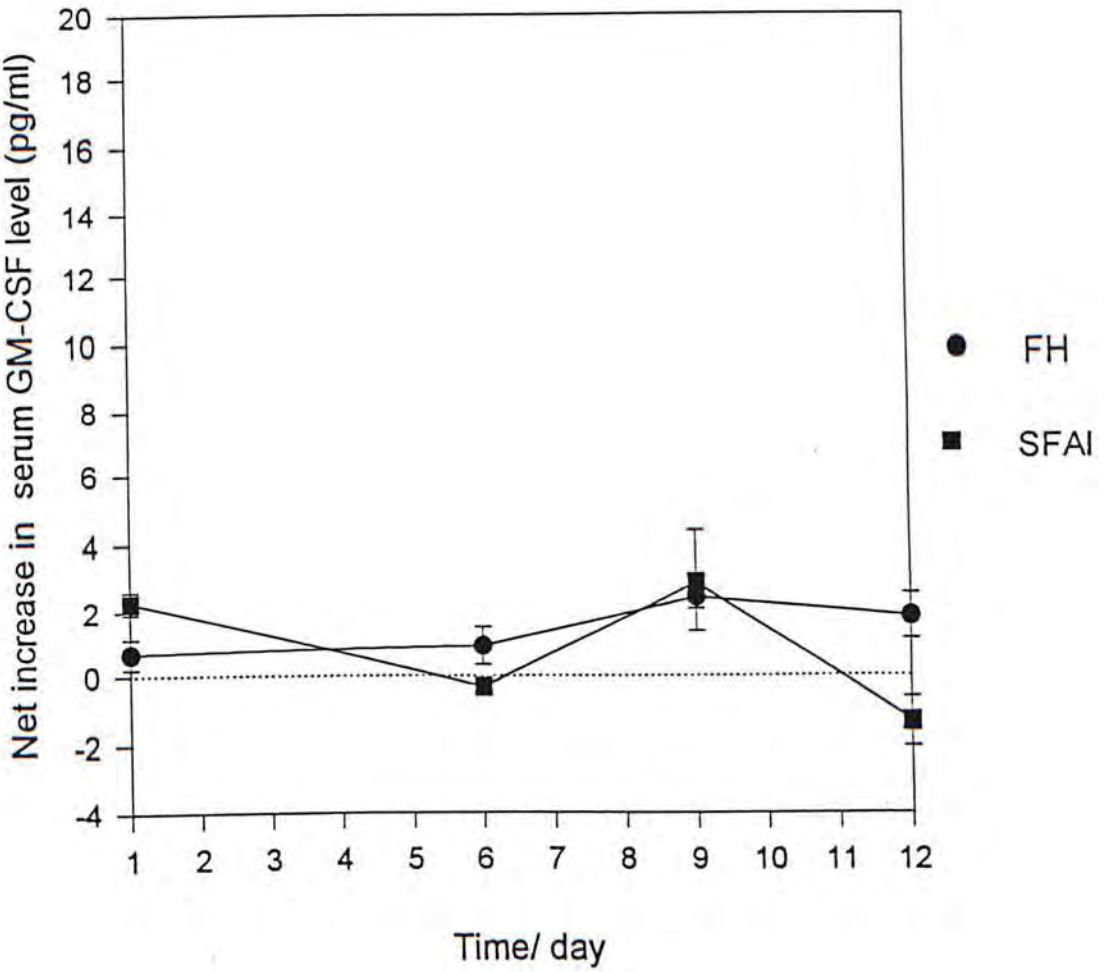


Fig. 5.15 Male ICR albino mice were injected intraperitoneally either with NaCl, FH or SFAl on day 0, 1, 2 and 3. On day 1, 6, 9 and 12, 5 mice were selected from each group and serum was obtained from the mice. The serum collected was tested for GM-CSF level by murine GM-CSF ELISA kit. Vertical bar represents one standard error.

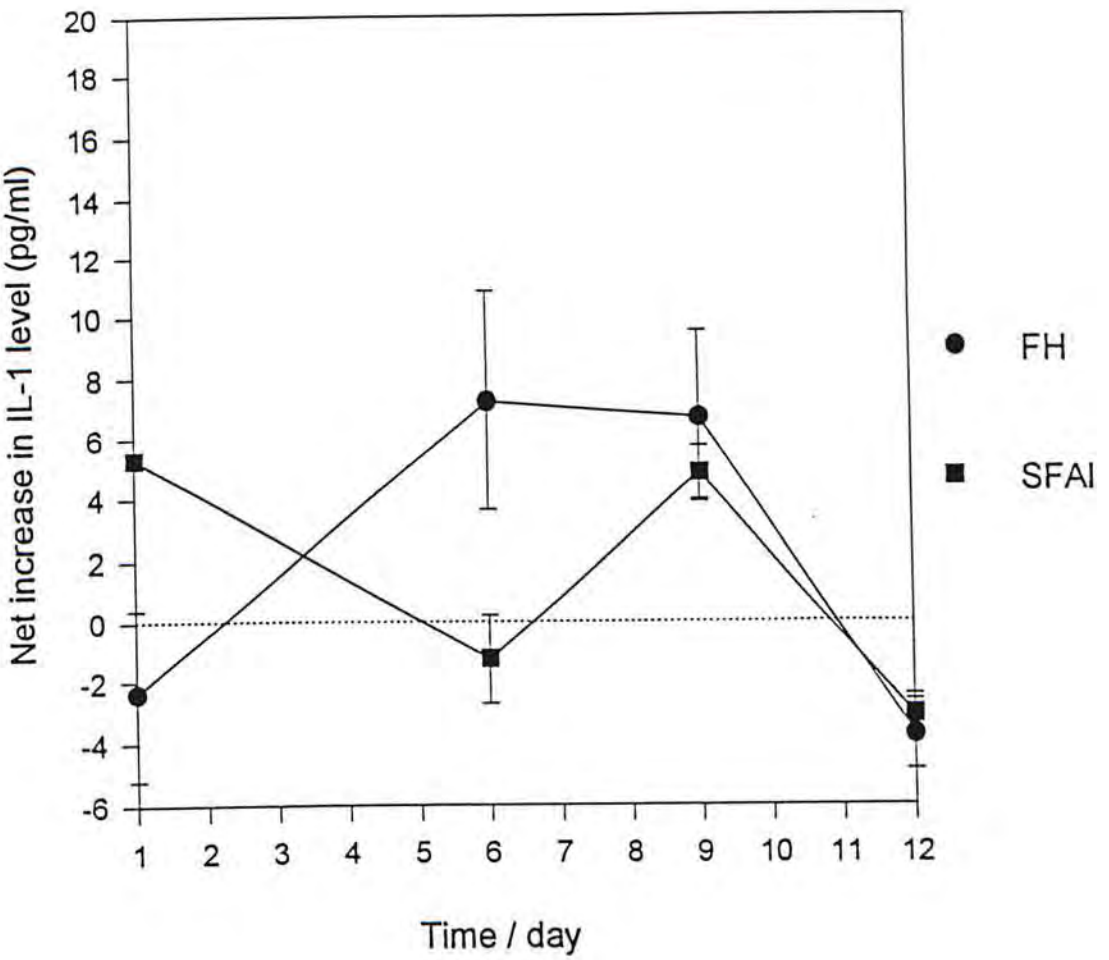


Fig. 5.16 Male ICR albino mice were injected intraperitoneally either with NaCl, FH or SFAI on day 0, 1, 2 and 3. On day 1, 6, 9 and 12, 5 mice were selected from each group and serum was obtained from the mice. The serum collected was tested for IL-1 α level by murine IL-1 α ELISA kit. Vertical bar represents one standard error.

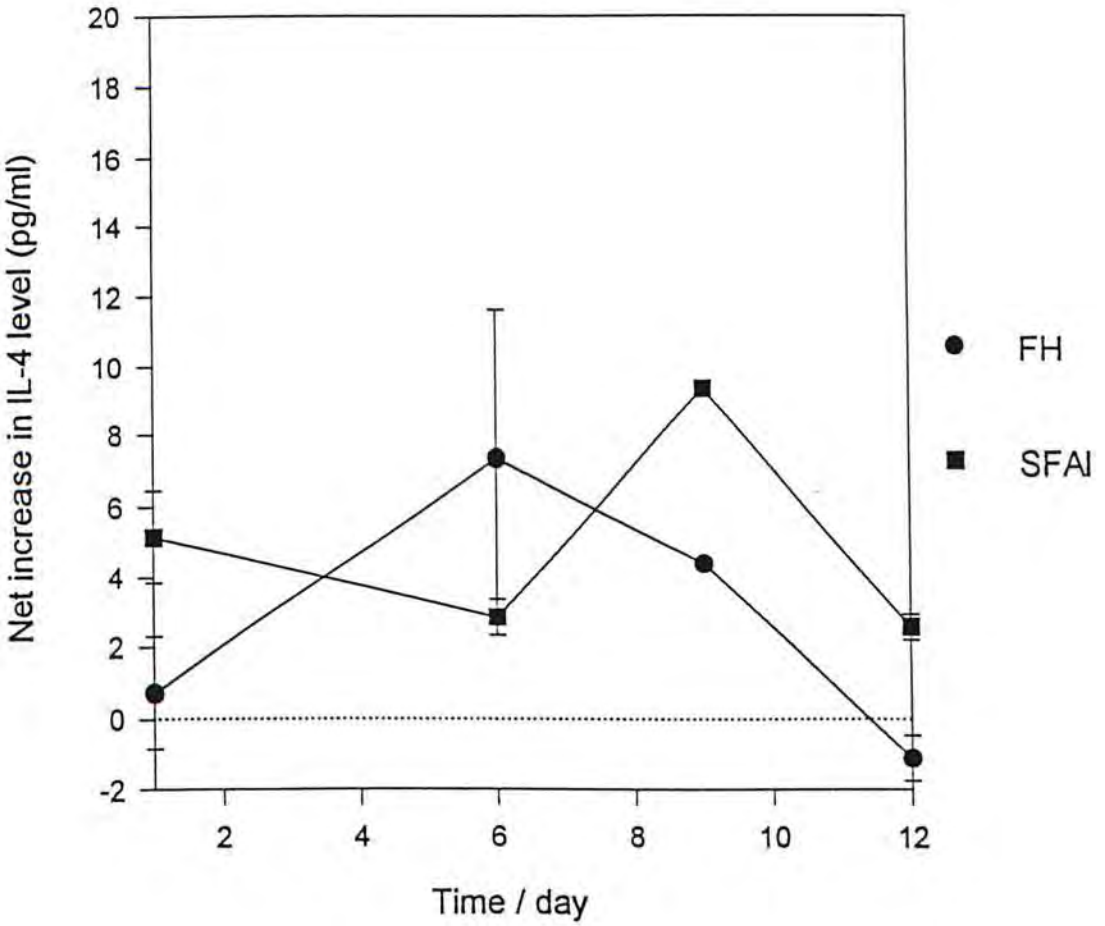


Fig. 5.17 Male ICR albino mice were injected intraperitoneally either with NaCl, FH or SFAI on day 0, 1, 2 and 3. On day 1, 6, 9 and 12, 5 mice were selected from each group and serum was obtained from the mice. The serum collected was tested for IL-4 level by murine IL-4 ELISA kit. Vertical bar represents one standard error.

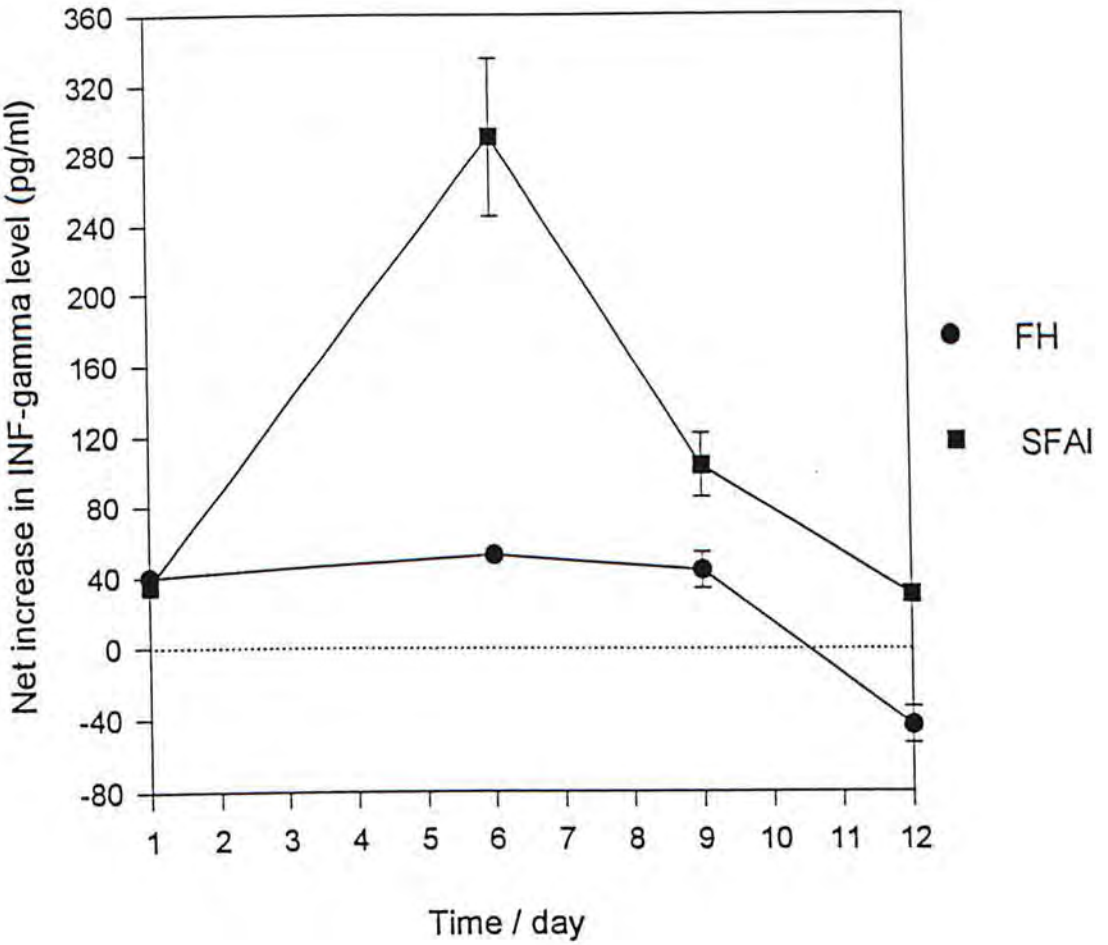


Fig. 5.18 Male ICR albino mice were injected intraperitoneally either with NaCl, FH or SFAl on day 0, 1, 2 and 3. On day 1, 6, 9 and 12, 5 mice were selected from each group and serum was obtained from the mice. The serum collected was tested for INF-γ level by murine INF-γ ELISA kit. Vertical bar represents one standard error.

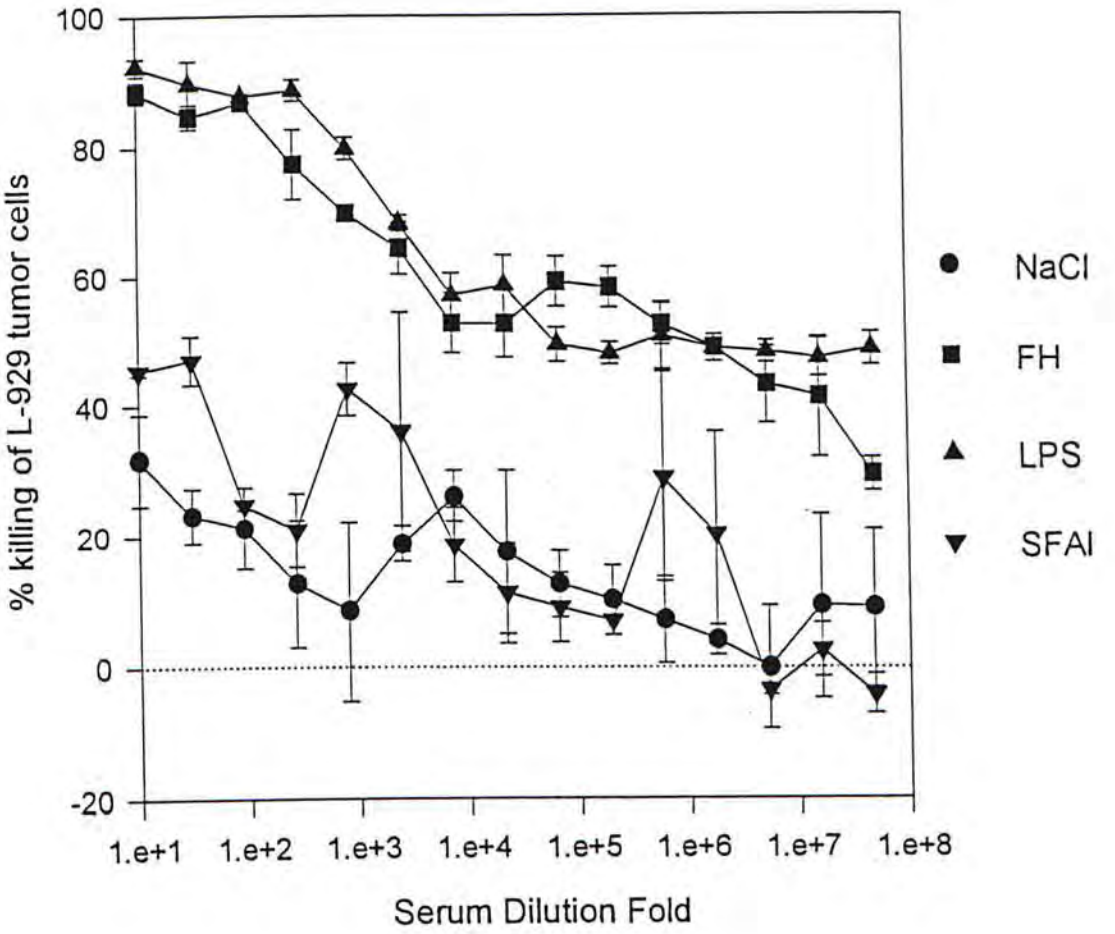


Fig. 5.19 The effect of FH and SFAI on murine serum TNF level. Mice were injected intravenously with either NaCl, FH, SFAI or LPS. 1 hr after treatment, blood was obtained from the mice. Serum prepared from the blood was diluted, with medium containing cycloheximide, to various concentrations. The diluted serum, 100 µl was incubated with 4×10^4 L-929 cells to test the TNF level. The amount of L-929 cells that survived the incubation was monitored with neutral red dye. Vertical bar represents one standard error.

5.3.2 Effect of FH and SFAl on the Augmentation of Murine Serum SRBC Lysing Factor Level

Male ICR albino mice were injected with SRBC intraperitoneally on day 0. The mice were also injected either with NaCl, FH or SFAl on day -2, 0 and 2. Serum obtained from the mice on day 6 was tested for SRBC lysing factor level. The SRBC lysing factor level was monitored by the degree of SRBC lysis. As clearly shown in Fig. 5.20, serum obtained from ICR albino mice treated with SFAl caused significantly more SRBC lysis with respect to control. However, serum obtained from ICR albino mice treated with FH just increased the SRBC lysis slightly when compared with control.

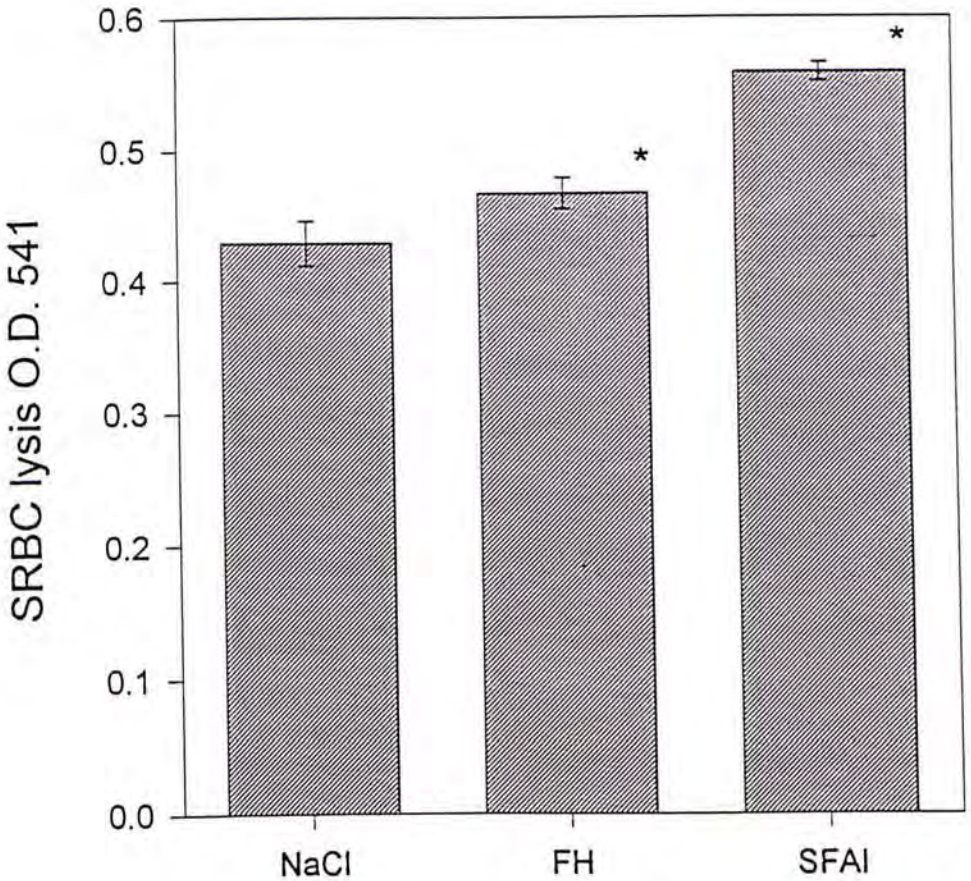


Fig. 5.20 The effect of FH and SFAI on the augmentation of mouse serum SRBC lysing factor (complement) level. Mice, immunised with SRBC on day 0, were either injected with NaCl, FH or SFAI intraperitoneally on day -2, 0 and 2. Serum was obtained from the mice on day 6 and the SRBC lysing factor level of the serum was monitored by the ability of the serum to lyse sensitised SRBC. The degree of SRBC lysis was measured by measuring absorbance at 541 nm.

Discussion

The results of this chapter summarised the effect of FH and SFAl on some important immune cells—B-, T-cells and macrophages. In the *in vitro* system, it is clear that only FH possess the ability to cause blastic transformation of murine splenic lymphocytes. Even the mitogenic activity of FH is small but still significant. The mitogenic activity of FH was not affected much by the present of PMB. Since PMB can bind to lipid A of LPS and abolishes the mitogenic activity of LPS (Jacobs, 1977), the blastic transformation ability of FH in the *in vitro* system should not be due to LPS contamination. When further tested, it was found that FH is a weak B-cell rather than a T-cell mitogen *in vitro*. Even though FH possesses some blastic transformation ability by itself alone, FH like SFAl cannot potentiate the mitogenic activity of some powerful mitogenic agent such as LPS and ConA *in vitro*. Furthermore, FH and SFAl also failed to increase the division of mouse bone marrow cells *in vitro*. In *in vivo* system, a different situation was observed, both FH and SFAl were found to possess the ability to increase the blastic transformation of mouse splenic lymphocytes. In SRBC immunised mice, the first antibody production was not affected by FH and SFAl. This implied that FH and SFAl lack the capacity to stimulate B-cell in the *in vivo* system.

Macrophages which play both regulator and effector function in *in vivo* system were not affected by FH but by SFAl in the *in vitro* system. However, in the *in vivo* system, the macrophages were activated by both FH and SFAl as there was a significant enhancement of *in vivo* phagocytic activity. Other than the enhancement of phagocytic activity, FH and SFAl also caused an increase in the migration of macrophages to the injection site *in vivo*. PEC containing macrophages, lymphocytes and other cell types when obtained from FH- and SFAl- treated mice did show an increased cytostatic activity against L-929 tumor cell *in vitro*. The increased cytostatic activity of the PEC inevitably enhance tumor killing process. Furthermore, PEC also exert a slight increase in Fc

receptor expression. The increased Fc receptor expression may enhance tumor killing through the antibody dependent cell cytotoxicity (ADCC) pathway. A greater number of Fc receptor on macrophage cell surface facilitated the binding of macrophages to tumor cells coated with antibody and thus enhanced subsequent tumor cell lysis.

There exist many types of cytokines of different biological functions. Cytokines act as chemical messengers for the communication of immune cells and other cell types. In fact, the effector phases of both natural and specific immunity are mediated by cytokines. In natural immunity, the effector cytokines (monokines) are mainly produced by mononuclear phagocytes. In specific immunity, most of the cytokines (lymphokines) are produced by T-lymphocytes. The lymphokines are mainly responsible for the regulation of the activation, growth and differentiation of lymphocyte population. Some of the lymphokines may also regulate and activate inflammatory cells, such as mononuclear phagocytes, neutrophils and eosinophils. The augmentation of the immune cells by FH and SFAI should involve an increase in the secretion of certain kinds of cytokines. Five types of cytokines; IL-1 α, IL-4, GM-CSF, TNF and IFN-γ , were selected for the investigation. It was found that only the serum TNF and IFN-γ level was affected by the administration of FH and SFAI.

Only FH has the capacity to trigger a higher serum TNF level. TNF was secreted from activated macrophages, T-cells, NK cells and mast cells. TNF kill tumor cells but not normal cells. The direct binding of TNF to tumor cells trigger lethal cellular events. TNF further enhance tumor killing by affecting the vasculature of tumor (cutting off nutrient supply to the tumor).

Both FH and SFAI have the capacity to increase serum IFN-γ level. The biological actions of IFN-γ support the idea that FH and SFAI when administered into mice activated T-cells and macrophages, and contributed to anti-tumor activity. IFN-γ was mainly secreted from activated T-cell but NK cells also have some serections. The secreted IFN-γ activated the mononuclear phagocyte, endothelial cell, NK cell, and increase the class I

and II MHC molecules of all cell types (Abbas, 1991). Mononuclear phagocyte and NK cell activated by IFN-γ acquire capacity to kill tumor cells effectively. The augmentation of the expression of class I MHC molecule may make the tumor more susceptible to attack achieved by immune effector mechanisms.

Other than serum cytokine level, the serum SRBC lysing factor level was also affected by FH and SFAI. SFAI did increase the serum SRBC lysing factor level of treated mice significantly. However, FH only increase the mouse serum SRBC lysing factor level just slightly. The increased SRBC lysing factor may be complement, which may play a role in the regression of tumor *in vivo*. Tumor cells that express particular antigens on the cell surface such as TSAs may sensitise the tumor bearing host to produce specific antibody direct against the tumor antigens. Tumor cells that were coated with the antibody were susceptible to lysis by complement through the classical complement pathway.

The Anti-tumor Activities of *Flammulina velutipes*

CHAPTER SIX

THE ANTITUMOR ACTIVITIES OF *Flammulina velutipes*

Introduction

Antitumor polysaccharides have been obtained from diverse sources, such as yeasts, fungi, lichens, bacteria and plants. The most active polysaccharides are β-(1→3)-D-glucan isolated from fungi and lichens (Chihara *et al*, 1970; Shibata *et al*, 1968). The structure and anti-tumor activity of the glucans vary from source to source. Lentinan (LNT), a famous β-(1→3)-D-glucan extracted from *Lentinus edodes*, is very effective against mouse Sc-180 (Chihara *et al*, 1970), whereas the related pachyman is inactive but gains activity after partial Smith degradation which remove the side branches. In contrast to pachyman, schizophyllan (SPG) from *Schizophyllum commune* (Yamamoto *et al*, 1981) and zymosan from yeast cell wall (Brander *et al*, 1958) are highly branched β-(1→3)-D-glucan, which show anti-tumor activity. Despite of the variations in the structure of the active β-(1→3)-D-glucans, mechanism involved in the antitumor action of the polysaccharides against various implantable tumor cells is generally considered to be host mediated and not directly cytocidal (Bradner *et al*, 1958; Chihara *et al*, 1970).

Results

6.1 *IN VITRO* ANTI-TUMOR ACTIVITY OF FH AND SFAI

PU5-1.8 and Sc-180 tumor cells were cultured with FH or SFAI at various concentration (25 μg/ml, 50 μg/ml and 100 μg/ml). The direct toxic effect of FH and SFAI

on the tumor cells were monitored by both thymidine incorporation and MTT dye. The results are shown in Fig. 6.1, 6.2, 6.3 and 6.4 respectively. The results of thymidine incorporation agreed with that of MTT dye. For both FH and SFAl, they showed little cytotoxic effect on the tumor cell lines. As the concentration of the polysaccharides increased, the percentage of tumor killing increased. Maximum killing usually attained at concentration beyond 50 μg/ml.

6.2 Effect of FH and SFAl on the Growth of Murine Transplantable Tumors

PU5-1.8 tumor cells (a syngeneic tumor) were grown in female BALB/c mice and Sc-180 tumor cells (an allogeneic tumor) were grown in male ICR albino mice. In case of PU5-1.8 tumor cells, the syngeneic tumor were injected into the peritoneal cavity of the female BALB/c mice on day 0. The regression of PU5-1.8 tumor was monitored by counting the number of viable tumor cells of the peritoneal wash on day 7. The mice were either injected with NaCl, FH, FAl, SFAl or Yeast cell wall (YW) on day -2, -1, 0, 1, and 2. The results were shown in Fig. 6.5. As shown in the figure, the injection of FH , but not FAl or SFAl , at 500 μg/mouse reduced the tumor growth significantly. For Sc-180 tumor, the allogeneic tumor cells were injected into male ICR albino mice subcutaneously. The mice were also injected either with NaCl, FH, FAl, SFAl, IFAl or α-cellulose on day 1, 3, 5, 7 and 9. Thirty-five days after the tumor injection, the regression of the tumor was monitored by weighting the solid tumor. The results are shown in Table 6.1. The injection of FH, FAl and SFAl caused regression of the tumor significantly but not the injection of IFAl or α-cellulose. The regression potency of FH, FAl and SFAl was similar (93, 98 and 94 % respectively).

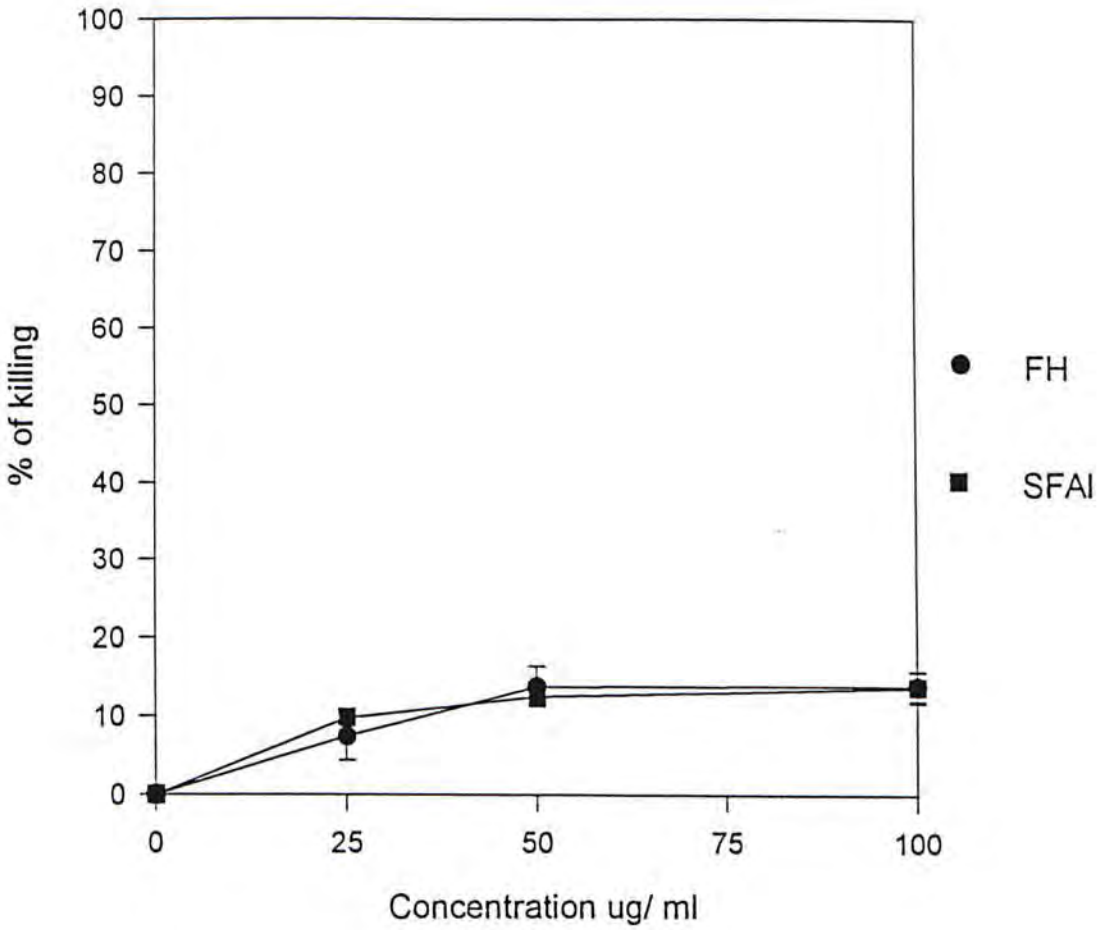


Fig. 6.1 The *in vitro* cytotoxic effect of FH and SFAI on PU5-1.8 tumor cells. Various concentrations of FH and SFAI were cultured with 10^5 tumor cells for 48 hr. After the incubation, the cells were pulsed with 5 μCi ^{3}H-TdR for 6 hr. and radioactivity incorporated was determined. Vertical bar represents one standard error.

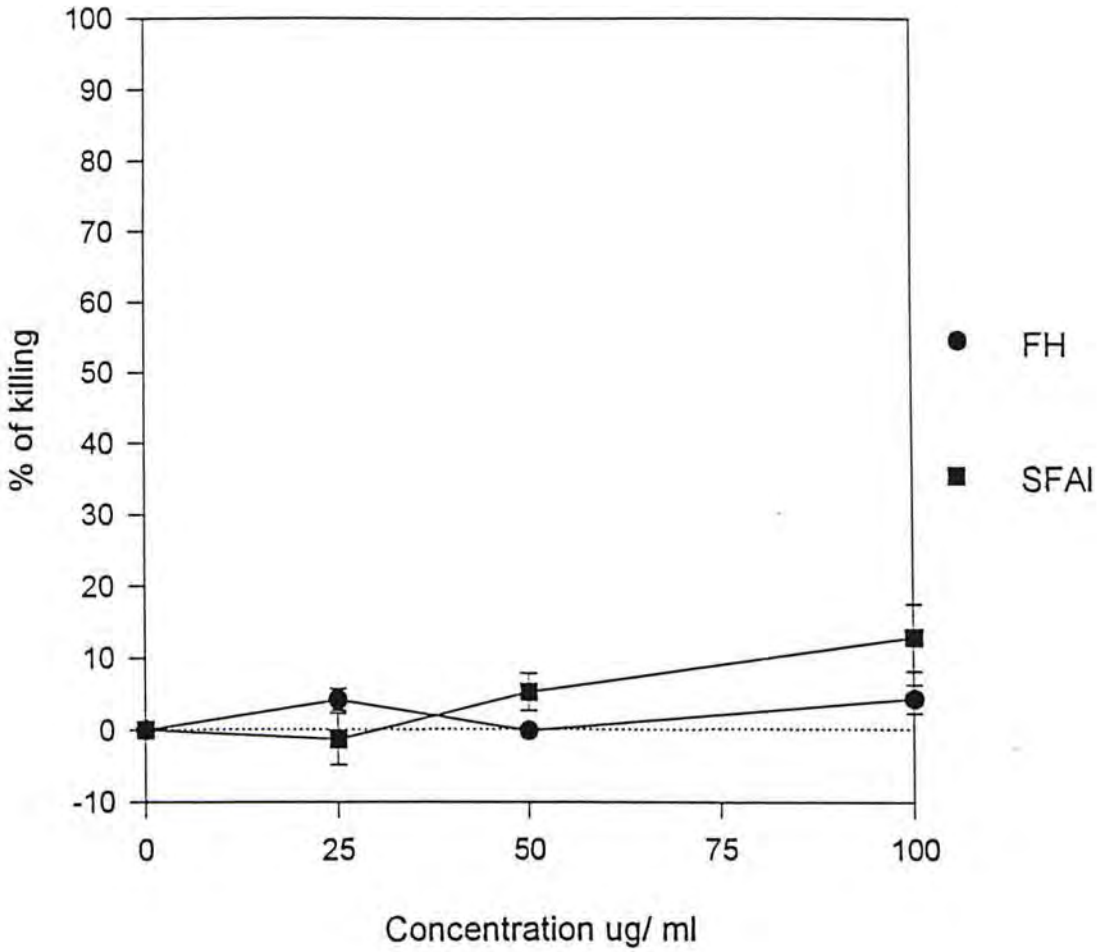


Fig. 6.2 The *in vitro* cytotoxic effect of FH and SFAl on PU5-1.8 tumor cells. Various concentrations of FH and SFAl were cultured with 10^5 tumor cells for 48 hr. After the incubation, 30 µl (5 mg/ml) MTT solution was added and incubated for further 1 hr. at 37 °C. Any blue formazan formed was solubilised by acidified isopropanol. Absorbance was measured at 540 nm. Vertical bar represents one standard error.

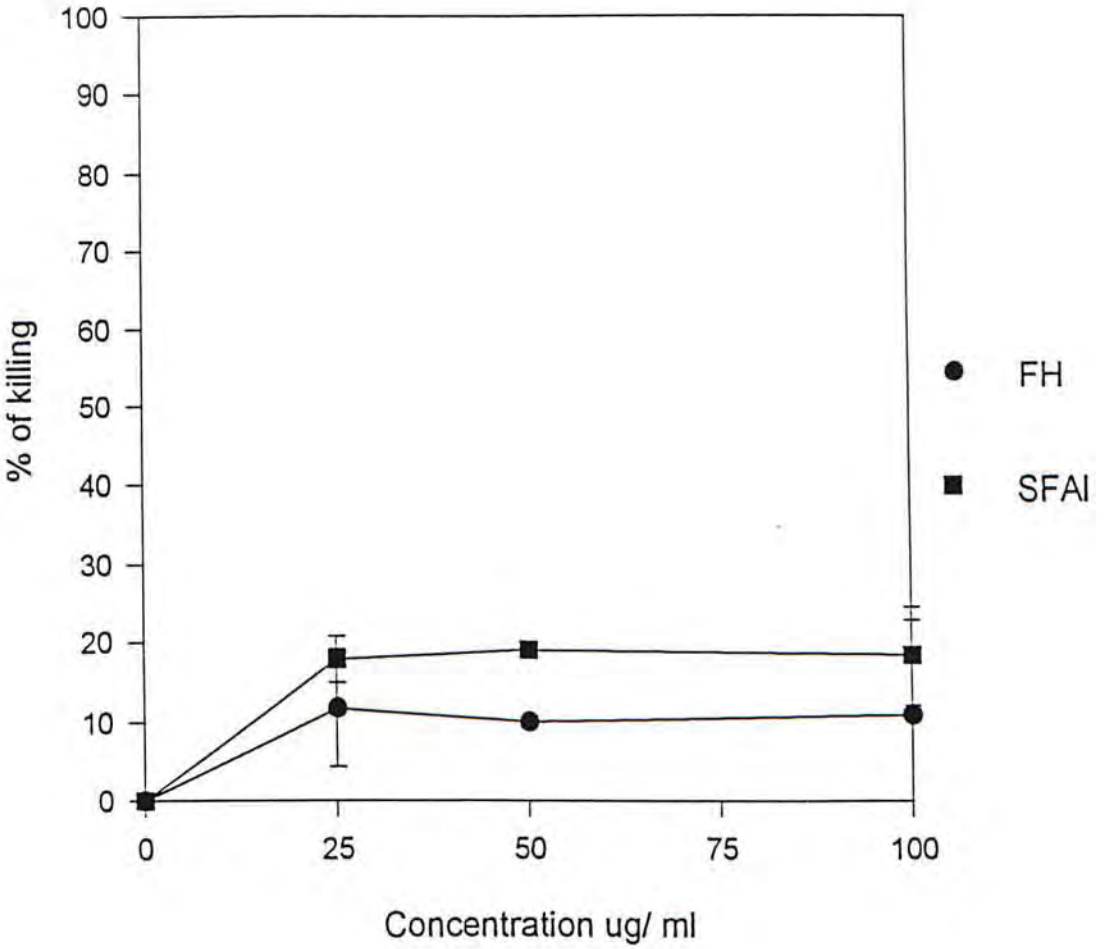


Fig. 6.3 The *in vitro* cytotoxic effect of FH and SFAl on Sc-180 tumor cells. Various concentrations of FH and SFAl were cultured with 10^5 tumor cells for 48 hr. After the incubation, the cells were pulsed with 5 μCi ^{3}H-TdR for 6 hr. and radioactivity incorporated was determined. Vertical bar represents one standard error.

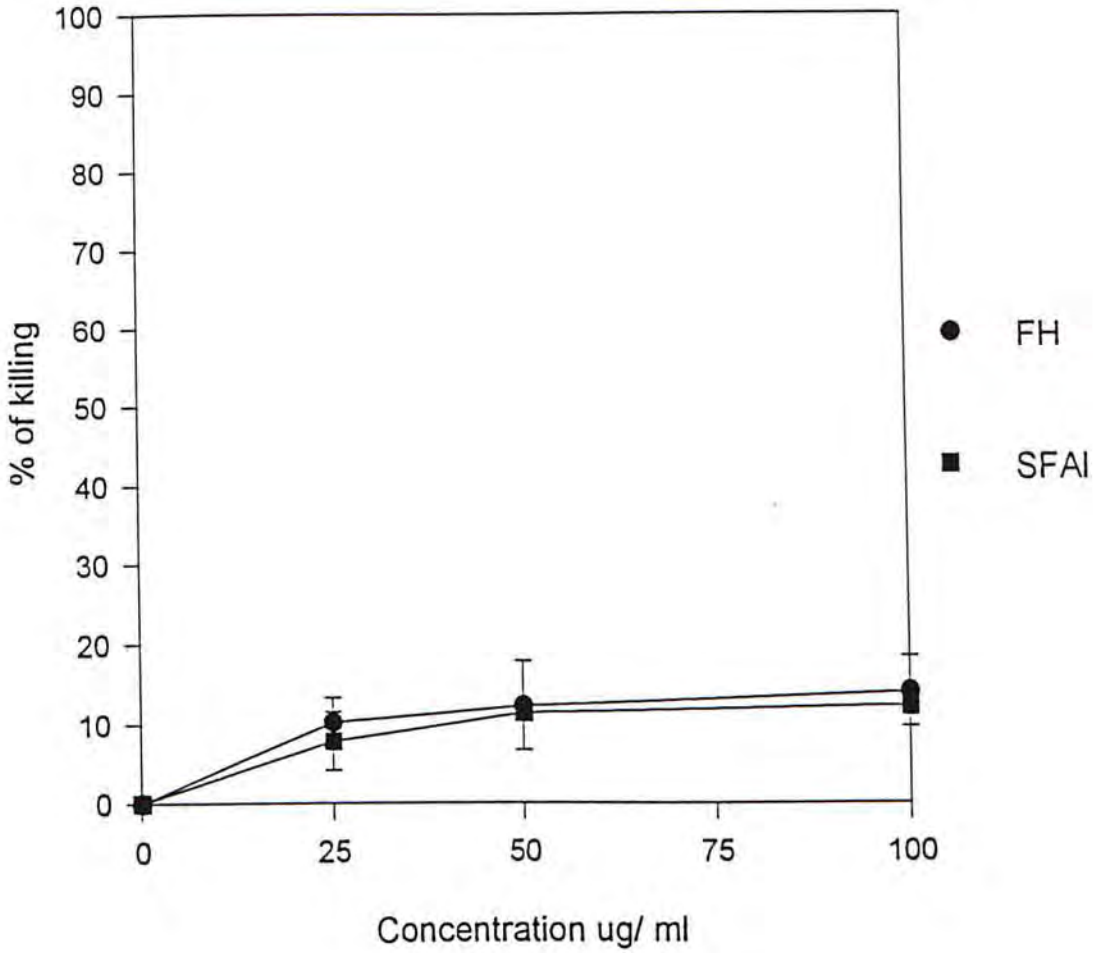


Fig. 6.4 The *in vitro* cytotoxic effect of FH and SFAl on Sc-180 tumor cells. Various concentrations of FH and SFAl were cultured with 10^5 tumor cells for 48 hr. After the incubation, 30 µl (5 mg/ml) MTT solution was added and incubated for further 1 hr. at 37 °C. Any blue formazan formed was solubilised by acidified isopropanol. Absorbance was measured at 540 nm. Vertical bar represents one standard error.

Table 6.1 *In vivo* antitumor activity of different polysaccharide fractions isolated from *Flammulina velutipes*.

Sample	Dose (mg/ kg)	Inhibition (%)	Complete regression	Significance (P<)
NaCl	/	/	0/6	/
FH	15	93.1 ± 10.1	3/6	0.05
FAl	15	97.8 ± 2.9	3/6	0.05
SFAl	15	94.1 ± 9.1	4/7	0.05
IFAl	15	5.0 ± 34.2	0/6	N.S.
α-cellulose	15	-24.7 ± 40.5	0/5	N.S.

Sarcoma 180 tumors were implanted subcutaneously in male ICR albino mice on day 0. The mice were also injected either with NaCl, FH, FAl, SFAl, IFAl or α-cellulose. Mice with no noticeable size of tumor, 20 days after tumor inoculation, were removed. Thirty days after tumor injection, tumor was excised and weighted.

N.S. means not significant at $p<0.05$.

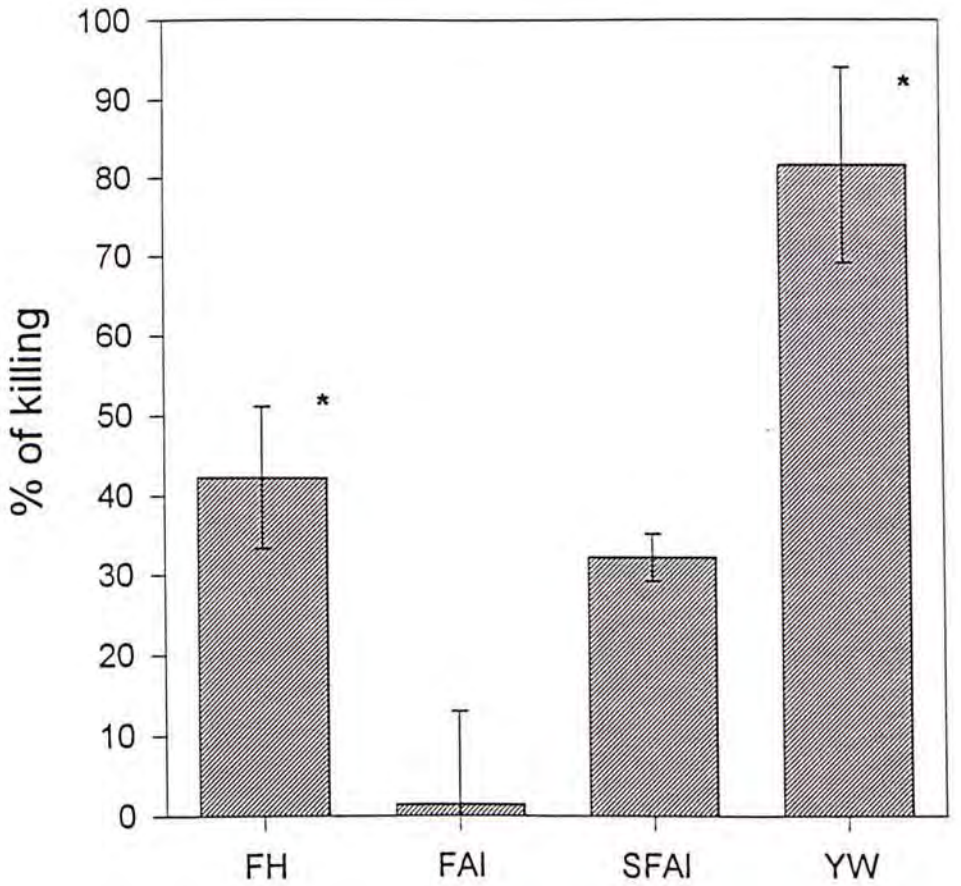


Fig. 6.5 The effect of FH and SFAI on the growth of PU5-1.8 tumor *in vivo*. Seven day old PU5-1.8 tumor cells, 10^6 were injected into the peritoneal cavity of female BALB/c mice on day 0. The mice were also treated with either NaCl, FH, FAI or SFAI on day -2, -1, 0, 1 and 2. On day 7, the number of viable PU5-1.8 tumor cells was counted by the tryphan blue dye exclusion method. The * mark means that the experimental value was significantly different from that of control at $p < 0.05$. Vertical bar represents one standard error.

Discussion

In our previous work, FH and SFAl were shown to be non-toxic to normal cells. The lack of direct cytocidal activity of FH and SFAl on tumor cells was demonstrated by culturing PU5-1.8 and Sc-180 tumor cells with FH and SFAl *in vitro*. In contrast to the *in vitro* studies, the administration of FH and SFAl into tumor bearing mice caused regression of the tumor. The regression of the tumor should not be due to a direct cytocidal effect of FH and SFAl, as the *in vitro* studies suggested, but very likely be due to the enhancement of host mediated killing mechanisms—the immune system.

General Discussion

CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Flammulina velutipes is an edible mushroom which is a popular foodstuff in Oriental countries. This type of fungus has no known medicinal purposes in traditional Chinese medicine. However, like other edible mushrooms, the β-(1→3)-D-glucans extracted from the mushrooms were active against murine syngeneic and allogeneic tumors in mice.

The FH and SFAl fractions extracted from *Flammulina velutipes* were found to contain mainly polysaccharides which have mainly β-(1→3)-D-linkage and other minor linkage(s) in the polymer. The minor linkage as revealed by the periodate oxidation is very likely to be β-(1→6)-D-linkage. If there are really 40 % of glucose in the fractions involved in the β-(1→6)-D-linkage, it is very likely that the primary structure of FH and SFAl is similar to structure shows in Fig. 7.1—a structure of (1→3)-D-glucan containing 3 β-(1→3)-D-glucose residues in a linear chain and 2 β-(1→3)-D-glucose residues forming a single branch, or similar to the structure of the water-soluble D-glucan extracted from *Auricularia auricula-judae* (Misaki *et al*, 1981; Ukai *et al*, 1983) as shown in Fig. 7.2—a structure of (1→3)-D-glucan consisting 3 β-(1→3)-D-glucose residues in a linear chain and 2 β-(1→6)-D-glucopyranoside branches. The minor linkage or the actual linkage can be elucidated by methylation analysis which has not been done in this project. As mentioned before, the Congo Red method just provided a preliminary study of the higher structure of the β-(1→3)-D-glucan(s) in FH and SFAl. β-(1→3)-D-glucan(s) in FH was found to have a triple helical structure but SFAl was found to have a single helical structure. In order to have a clearer picture of the structures, data from IR, NMR and even X-ray crystallographic analysis should be obtained.

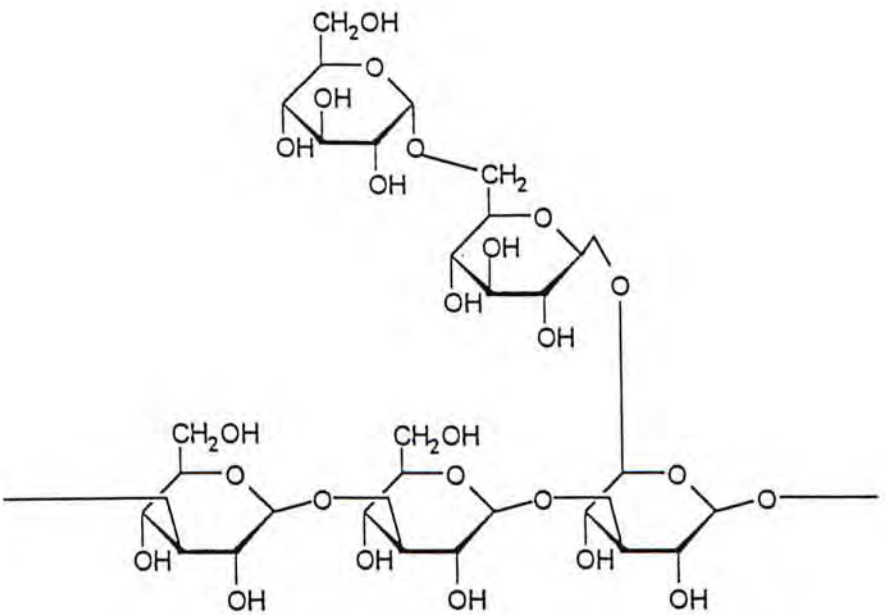


Fig. 7.1 Chemical structure of a D-glucan containing 3 β-(1→3)-D-glucose residues in a linear chain and 2 β-(1→3)-D-glucose residues forming a single branch.

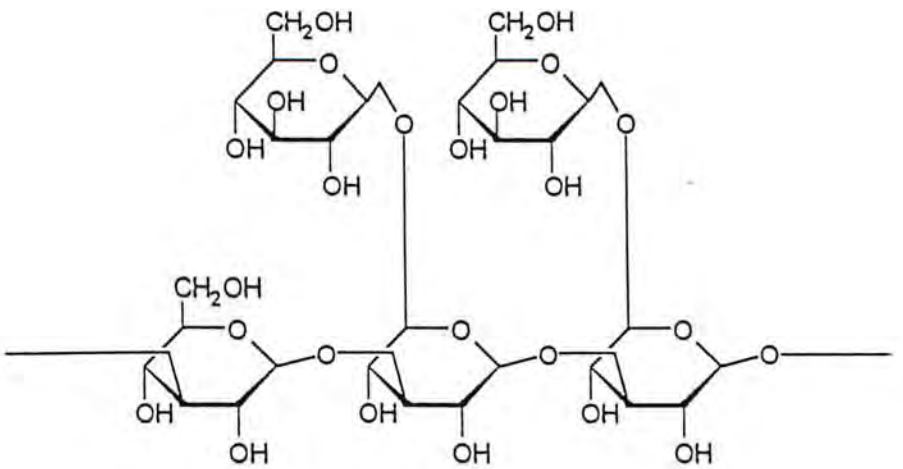


Fig. 7.2 Chemical structure of a water-soluble D-glucan extracted from *Auricularia auricula-judae.*

The purity of the fractions especially the FH fraction was challenged by the gel permeation chromatographic separation using G-200. Indeed, the separation profile of FH showed a major and a minor peak. The two fractions, the major and the minor peak, of FH were not separated for bioassay or further analysis. The chromatographic separation require FH fraction to be treated with alkaline to reduce the viscosity of FH but at the same time disrupted the higher structure of FH. From chemical analysis, the polysaccharides of FH were very likely to have no charge and thus ion exchange chromatography was not suitable for separation purpose. It seems that fractional precipitation or ultracentrifugation may be ideal for the further purification of FH and SFAl.

In general, FH and SFAl were both not very active in *in vitro* system. However, in the *in vivo* system, FH and SFAl were found to be able to activate macrophage and lymphocyte. The type of lymphocytes to be activated was very likely to be T-lymphocyte as suggested by the VDH response (Aoki, 1984). In fact, FH and SFAl were demonstrated not able to enhance the B-lymphocyte function *in vivo*. Whether other immune cells such as NK , CTL etc. were activated by FH and SFAl require further investigation. FH and SFAl were found to be able to activate some effectors of the immune system of mice. It is still a question whether FH and SFAl have the capacity to activate the human immune system.

The activation of the immune effectors may lead to the secretion of TNF. FH was found to be able to trigger the release of a factortaht killed the tumor cells, which may be tumor necrosis factor (TNF), one hour after intraveneous injection. If the released killing factor was really TNF, FH can thus trigger TNF release by a single injection. This is quite uncommon. The release of TNF may involve three stages; the normal stage, the primed stage and the triggered stage. At normal stage, no transcription or translation of TNF occurred. At the primed stage, TNF gene is actively transcripted and the transcripted TNF mRNA is then translated to give precursor TNF. The translated precursor TNF

anchored on cell surface membrane and ready to be released. At triggered stage, stimulant elicits the processing of precursor TNF and releases of mature TNF. Most substances which can elicit TNF release by furnishing the triggered stage but not the primed stage. If the released factor is TNF, the action of FH may already exist at the primed stage. Therefore, no priming action (e.g. administration of zymosan as a primer) is required. Alternatively, the polysaccharide(s) of FH may serve as a primer as well. Zymosan, which is roughly composed of equal amount of β-(1→3)-D-glucan and mannan. The close resemblance of polysaccharide(s) of FH to β-(1→3)-D-glucan of zymosan may allow FH to furnish the priming and the triggering event.

FH and SFAI were found to be non-toxic to normal and tumor cells *in vitro*. However, FH and SFAI were highly tumoricidal *in vivo*. All the evidence so far suggested that FH and SFAI caused the regression of tumor *in vivo* by the enhancement of host mediated killing mechanisms rather than a direct cytocidal effect. The actual mechanism of the augmentation of the antitumor immunity was not known but it may be due to the antigenicity of the polysaccharides. Exogenous substances activate host immune system when entered the host body. Unlike proteins, the antigenicity of polysaccharides is rather low. In fact, polysaccharide polymer with highly regular structure and small molecular weight such as dextran of molecular weight below 50,000 do not induce antibody response in humans (Kabat, 1993). However, polysaccharide with highly regular structure but high molecular weight such as dextran of molecular weight over 90,000 have been shown to be antigenic in humans (Kabat, 1993). The extremely high molecular weight of the polysaccharides in the FH and SFAI fractions support the postulation that the injection of FH and SFAI into tumor bearing mice activated the immune system of the host. The activation may involve the processing of the possible antigenic polysaccharides. The activated immune cells not only remove the invading antigenic polysaccharides but also help furnishing tumor cells killing. Alternatively , FH and SFAI may augment the antitumor immunity through non-specific activation of the host immune system. The

administration of the FH and SFAI into tumor bearing host stimulates the host immune response non-specifically but activated immune cells contribute to tumor cells killing in tumor bearing host. In this case, FH and SFAI may not act as antigenic polysaccharides. For example, FH and SFAI were not processed before they can activate the immune system. In fact, no evidence suggested that there exist β-(1→3)-D-glucanase, which can digest β-(1→3)-D-glucan, in mammalian cells. Therefore, there may not exist any processing mechanisms to process the administered FH and SFAI. Furthermore, Czop and Austen (1985) reported a β-D-glucan inhibitable receptor on human monocytes. The investigation of Czop found that the binding β-D-glucan to the β-D-glucan receptor elicits monocytes to metabolise endogenous arachidonic acid to substantial amount of luekotriene B_4 and thus cellular events. The experiment of Czop was done in *in vitro* conditions and no processing of glucan exist before the β-D-glucan applied to the monocytes.

No matter which pathways is involved in the augmentation of the antitumor immunity. The administration of FH and SFAI into tumor bearing mice may activate macrophages and T-lymphocytes. The activated macrophages may furnish tumor cell killing through direct interaction with tumor cells or the secretion of TNF and complement. The direct binding of TNF to tumor cells trigger lethal cellular events. TNF further enhance tumor cells killing by cutting the blood supply of tumor mass. Complement secreted by macrophage may assist tumor cells killing through complement cell lysis pathway. The activation of T-cells may lead to the secretion of interferon-gamma (INF-γ) . The secreted INF-γ may help furnishing tumor cells killing through several means. Firstly, INF-γ can augment the action of TNF and thus tumor cells killing. Secondly, INF-γ can promote the differentiation of B- and T- lymphocytes directly. The differentiated B- and T-lymphocytes may regulate antitumor effectors or be effector themselves. Finally, INF-γ can augment the expression of Class I and II MHC molecule. The increase class II MHC molecule may enhance the recognition of tumor antigens by the

immune system and thus enhance tumor cells killing. An increase in the expression of class I MHC molecule also help furnishing tumor cells killing. The increased expression of class I MHC molecule may make tumor cells more susceptible to attack achieved by immune effector such as cytolytic T-lymphocytes.

To be a useful antitumor agent for clinical purpose, FH and SFAI should be shown to be active anti-tumor agent in human. Furthermore, the usefulness also depends on whether the fractions were active against various type of cancer. All these require further investigations. Other than the use of FH and SFAI as a potential anti-tumor agent, the immunopotentiating activities of FH and SFAI suggest that FH and SFAI may have potential use in the therapy of some viral, bacterial, fungal and even highly pathogenic parasitic diseases such as leishmaniasis, filariasis, toxoplasmosis etc..

The hydroxyl groups of the glucose monomers of the glucan can easily be substituted with groups such as sulfate, acetate, amino, carboxyl methyl etc.. It is interesting to chemically modify FH and SFAI by adding substituent group to the polysaccharides. Furthermore, the effect of debranching and digesting the polysaccharides on the immunomodulatory and antitumor activity will remain an interesting topic for future investigations.

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